

The Effects of Omega 3 Supplementation  
on Markers of Obesity and Endothelial Function  
in Healthy Subjects

by

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## ABSTRACT

The hormone leptin is an important regulator of body weight and energy balance, while nitric oxide (NO) produced in the blood vessels is beneficial for preventing disease-induced impaired vasodilation and hypertension. Elevations in the free radical superoxide can result in impaired vasodilation through scavenging of NO. Omega 3 is a polyunsaturated fatty acid that is beneficial at reducing body weight and in lowering many cardiovascular risk factors like atherosclerosis. The present study was designed to examine the change in plasma concentrations of leptin, nitric oxide, and the antioxidant superoxide dismutase in addition to examining the association between leptin and NO in healthy normal weight adult female subjects before and following omega 3 intakes. Participants were randomly assigned to either a fish oil group (600 mg per day) or a control group (1000 mg of coconut oil per day) for 8 weeks. Results showed no significant difference in the percent change of leptin over the 8 week supplementation period for either group ( $15.3\pm 31.9$  for fish oil group,  $7.83\pm 27$  for control group;  $p=0.763$ ). The percent change in NO was similarly not significantly altered in either group ( $-1.97\pm 22$  decline in fish oil group,  $11.8\pm 53.9$  in control group;  $p=0.960$ ). Likewise, the percent change in superoxide dismutase for each group was not significant following 8 weeks of supplementation (fish oil group:  $11.94\pm 20.94$ ; control group:  $11.8\pm 53.9$ ;  $p=0.362$ ). The Pearson correlation co-efficient comparing the percent change of both leptin and NO was  $r^2 = -0.251$  demonstrating a mildly negative, albeit insignificant, relationship between these factors. Together, these findings suggest that daily supplementation with 600 mg omega 3 in healthy females is not beneficial for

improving these cardiovascular risk markers. Future studies in this area should include male subjects as well as overweight subjects with larger doses of fish oil that are equivalent to three or more servings per week. The importance of gender cannot be underestimated since estrogen has protective effects in the vasculature of females that may have masked any further protective effects of the fish oil. In addition, overweight individuals are often leptin-resistant and develop impaired vasodilation resulting from superoxide-mediated scavenging of nitric oxide. Therefore, the reported antioxidant and weight loss properties of omega 3 supplementation may greatly benefit overweight individuals.

I would like to dedicate this to my wife. You are the love of my life, and you have walked this journey with me, supporting me every step of the way. I am grateful for you, and I thank you from the bottom of my heart.

To my sons, my source of hope and joy for the future. You are my vision and hope for tomorrow, and the day after that, and the day after that.

To my mother , the woman who gave me life , the one who loved me , protected me , inspired me and shaped my character and future .Thank you for being a strong tree who sheltered and protected me in the glare of life's harsh realities .

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## CHAPTER 1

### INTRODUCTION

It is estimated that more than one third of adults and almost 17% of youth in the United States of America are obese. Approximately 41 million women and 37 million men ages 20 and over were obese in 2009–2010<sup>1</sup>. Among children and adolescents aged 2–19, there are more than 5 million girls and approximately 7 million boys who are obese. Given the high prevalence of obesity, obesity-related medical treatment accounts for nearly 10% of annual medical spending costs, estimated between \$147 and \$210 billion dollars each year<sup>2</sup>.

Obesity is considered as risk factor for many clinical conditions like diabetes, hypertension and cardiovascular disease (CVD). It is also related to the leading causes of death in the United States as heart disease and stroke are responsible for the mortality of about 750,000 people every year<sup>3</sup>. These devastating numbers indicate helpful strategies are desperately needed to assist in the battle against obesity and CVD.

Despite the availability of promising medical and surgical options in the management and prevention of obesity, the prevalence of obesity has not declined although cardiovascular mortality rates have declined in many high-income countries<sup>4-6</sup>. At the same time, cardiovascular deaths and disease have rapidly increased in low- and middle-income countries, as over 80% of all global deaths caused by CVD diseases is found in these countries. In addition, it is estimated that by 2030, over 23 million people will die from CVD every year globally<sup>7</sup>.

One approach to managing obesity is through dieting and changing lifestyle habits. This type of management is becoming more prevalent between patients and health providers<sup>8</sup>. Among these kinds of treatments available is the use of certain nutrients or foods to help to control or even prevent many health problems including obesity and CVD. Polyunsaturated fatty acids, especially omega 3, have been considered a good choice to meet this demand. In recent studies it was shown that increasing omega 3 can reduce body weight<sup>9</sup>. In addition, omega 3 consumption positively affects many CVD disease risk factors, including improvements in triglycerides, total cholesterol, and low density lipoprotein (LDL) cholesterol<sup>10</sup>. Moreover, many studies have shown that omega 3 supplementation is associated with a lower risk of developing coronary artery disease and hypertension as well as reduced adiposity and serum cholesterol<sup>11,13</sup>.

For better management and prevention of obesity and its negative impact on health, a proper understanding of the biological factors influencing body fat regulation are critical. Factors may include genetic and environmental effects as well as the role of adipose tissue as an active endocrine organ that produces different substances and hormones, one of which is leptin<sup>12</sup>. Leptin is secreted from the adipose tissue to maintain homeostatic control of adipose tissue mass in the body and body weight through regulating the activity of food intake, energy expenditure and metabolism<sup>12</sup>.

A prior study showed that a diet enriched in cholesterol and alpha linolenic acid (from flaxseeds) increased protein and mRNA expression of leptin in the adipose tissue of healthy rabbits<sup>16</sup>.

It is also important to understand the role of nitric oxide (NO) in the process of endothelium-dependent vascular relaxation as well as the scavenging of reactive oxygen species that develop during states of vascular stress and inflammation that accompany chronic pathological conditions like atherosclerosis, obesity, and diabetes. Leptin hormone increases the production of NO by endothelial nitric oxide synthase enzyme in the endothelial cells of blood vessels<sup>271</sup> while superoxide dismutase enzyme increases the bioavailability of NO through reducing reactive oxygen species that scavenge NO<sup>14, 236</sup>.

Many studies have focused mainly on the behavior of leptin and NO in experimental animals including genetically-modified rodents with few studies conducted on humans. To our knowledge, few research studies have examined the relationship between omega 3, leptin and NO in healthy human subjects. Therefore, the primary aim of this study will focus on the effects of omega 3 supplementation on NO and leptin as well as antioxidant superoxide dismutase which can improve NO bioavailability in healthy participants. It is predicted that omega 3 supplementation in healthy subjects will increase the bioavailability of NO and decreased super oxide radicals through increasing super oxide dismutase activity<sup>14-15</sup>. It is additionally predicted that omega 3 supplementation may increase leptin concentrations in healthy subjects. This study will lay the groundwork for future investigations regarding the efficacy of omega 3 supplementation for prevention of obesity and endothelial dysfunction.

## **Purpose of Study**

The primary purpose of this study is to compare the plasma concentrations of leptin in healthy adult women before and after omega 3 supplementation (600mg per day for eight weeks). A secondary purpose of this study is to compare the plasma concentrations of nitric oxide following the same dietary protocol. The tertiary purpose is to compare the plasma concentration of the antioxidant superoxide dismutase following the same dietary protocol. The quaternary purpose is to study the association between the concentration of leptin hormone and nitric oxide.

## **Aims and Hypotheses**

- **Primary Aim:**

To examine the change in leptin hormone in healthy adults before and after omega 3 supplementation (600mg per day for eight weeks).

- **Primary Hypothesis:**

Plasma leptin concentrations will not be changed after 8 weeks of daily omega 3 supplementation in healthy individuals compared to participants consuming coconut oil (placebo).

- **Secondary Aim:**

To examine the effect of omega 3 supplementation on plasma nitric oxide levels in healthy adults following daily omega 3 intake (600mg per day for eight weeks).

- Secondary Hypothesis:

There will be no change in plasma nitric oxide concentrations before and after 8 weeks of daily omega 3 supplementation in healthy adults compared to placebo-treated individuals.

- Tertiary Aim:

To examine the effect of omega 3 supplementation on plasma superoxide dismutase enzyme levels in healthy adults following daily omega 3 intake (600mg per day for eight weeks).

- Tertiary Hypothesis:

There will be no change in plasma superoxide dismutase concentrations before and after 8 weeks of daily omega 3 supplementation in healthy adults compared to placebo-treated individuals.

- Quaternary Aim:

To examine the association between serum leptin and NO levels in healthy subjects before and after 8 weeks of daily omega 3 supplementation compared to placebo treated individuals.

- Quaternary Hypothesis:

There will be a negative association between serum leptin and serum NO before and after 8 weeks of daily omega 3 supplementation in healthy adults compared to placebo treated individuals.



## **Limitations and Delimitations**

The main limitation in this study results from its being a secondary analysis of samples obtained from subjects that participated in a previous study under an already established study protocol. Another limitation is that all subjects who participated in the study were healthy females with no males or unhealthy subjects included.

Additional limitations are subject compliance to minimize the ingestion of omega 3 fatty acids and only consume either the placebo or fish oil capsules. All subjects were asked to complete a food frequency questionnaire to reduce this limitation by excluding subjects who reported normal consumption of 3.5 ounces of fish twice per week. To minimize the effects of physiological hormonal fluctuations, all measurements were performed at the same point in the menstrual cycle for each participant. Further limitations include the small sample size and short treatment time used for the study, as well as external factors such as stress. The results of this study are generalizable to individuals similar to the participants in the original study who were women aged 18-38 years without medical or health complaints at the time of study. The participants attended a large university in the southwestern U.S.

## CHAPTER 2

### REVIEW OF LITERATURE

#### **Leptin**

Obesity results from deviations in the status of energy balance from equilibrium (energy intake equal to energy expenditure) towards positive energy balance, with the extra energy stored as body fat. The regulation of energy expenditure and control of body weight is performed through the interaction of various peripheral and central systems with hormones like leptin serving as chemical messengers for communication between these systems. Leptin is a protein produced by adipose tissue that helps regulate the level of stored body fat by affecting satiety and energy intake. Moreover, altered signaling may disrupt this regulation thereby affecting energy balance.

The first assumptions that a hormone like leptin existed were based on the observation that animals and humans can, to an extent, maintain their weight for prolonged periods. Moreover, balancing energy intake and energy expenditure to maintain weight involves homeostatic controls<sup>12</sup>. This homeostasis is the result of interactions of biological factors such that any disturbance in body weight is corrected. The role of the central nervous system in the regulation of body weight was supported by evidence showing that lesions in the hypothalamus can cause obesity or leanness depending on the affected area (the ventromedial hypothalamus for obesity, and the lateral hypothalamus for leanness)<sup>17</sup>.

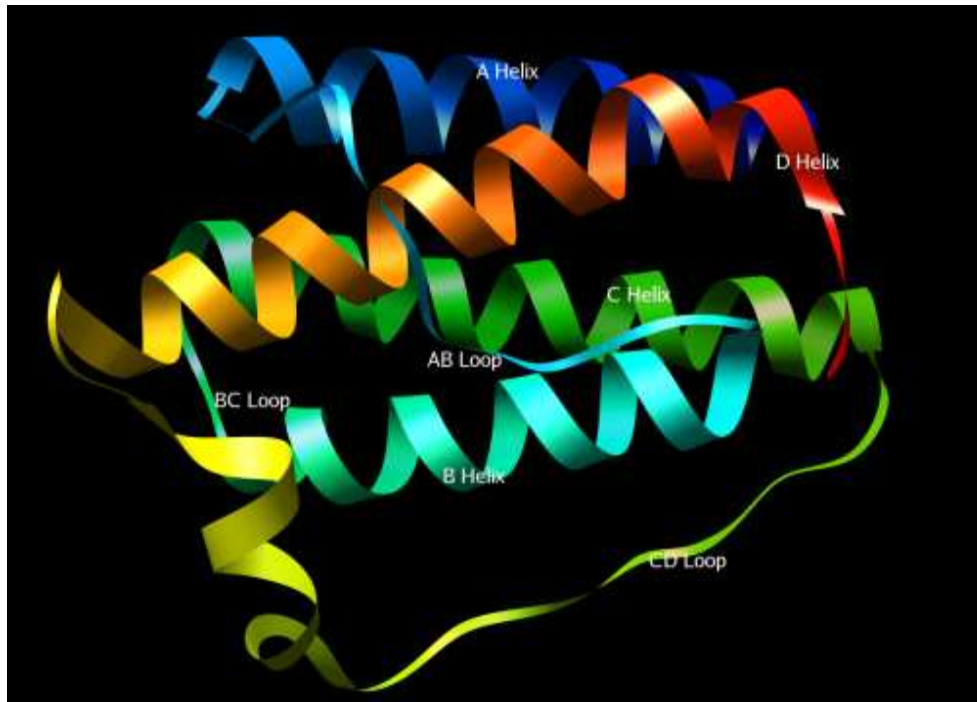
These observations led to the discovery that the hypothalamus is involved in a feedback loop wherein it receives peripheral messages from adipose tissue about an

organism's nutritional state and energy status<sup>18</sup>. Kennedy et al. proposed that the hypothalamus receives information from peripheral factors regarding the level of fat stores<sup>19</sup>. The hypothalamus, in turn, responds to this information by stimulating the modification of food intake.

Colman et al found that parabiosis (in which two mice were surgically united so the exchange of blood is possible between the two members) of normal mice with obese diabetic mice (db/db) caused the normal mice to become hypoglycemic and rapidly lose weight, eventually resulting in death from starvation and indicating that the diabetic mice produced what was thought to be a satiety factor that caused the normal mice to lose weight<sup>20</sup>. In contrast, parabiosis of obese mice (ob/ob) with normal mice did not change the feeding behavior of the normal partners. Instead, weight gain was suppressed in obese mice and after separation, the obese mice started to gain weight again suggesting that the obese mice were not producing a satiety factor and that they were responsive to that factor produced by normal mice. This blood factor produced by normal mice, and overproduced by db/db mice, can normalize ob/ob mice while no benefit from the introduction of that factor in db/db mice is observed. Moreover, the blood-borne elements produced by increased adiposity induced satiety in the normal rats<sup>20</sup>.

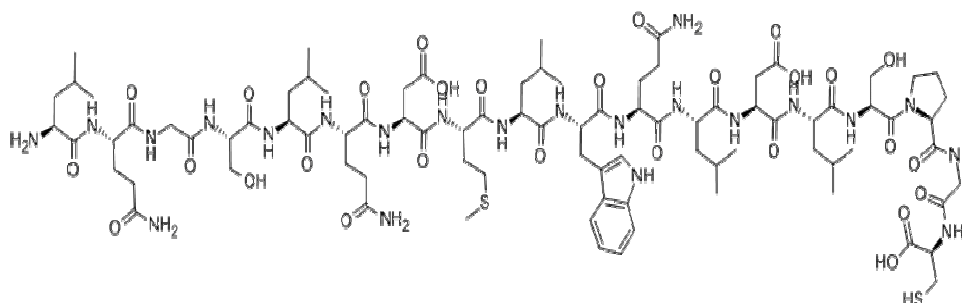
In another parabiosis experiment, Hervey et al discovered that introducing a hypothalamic lesion in one of the pair induced hyperphagia and obesity while the normal partner to become thin and ate less<sup>21</sup>. Moreover, when a lesion was introduced to the hypothalamus of thin mice, it caused hyperphagia indicating that a part of their hypothalamus is responsible for the overfeeding. These results provided evidence for a feedback control of food intake.

The hormone leptin (derived from leptos, a Greek word meaning “thin”) was finally identified in 1995, when the gene responsible for encoding leptin (ob gene) was cloned in 1994 with the help of positional cloning<sup>22</sup>. According to researchers, ob gene expression increases in adipose tissue from obese ob/ob rats as a result of overfeeding and lesions to the hypothalamus. Similarly, ob gene expression is increased in humans in proportion to the severity of obesity indicating a role for this gene in the development of obesity<sup>22</sup>.



*Figure 1. 3D structure of leptin hormone. Image from maptest.rutgers.edu*

Ob is an 18kb gene located at chromosome 7 and is composed of three exons and two introns<sup>23,24</sup>. This gene encodes the hormone leptin, a 166 amino acid protein whose biologically active site is the intra-chain disulfide bond. Leptin is a four helix bundle, which is similar to the structure of helical cytokines of the hematopoietin family<sup>24</sup>.



**Figure 2.** *Leptin Molecule.* Image from [www.guidechem.com](http://www.guidechem.com)

This protein is 84% similar to that of the rat indicating that it is well-conserved<sup>25</sup>. Leptin is produced and released by differentiated adipocytes. Since there is no evidence that the hormone is stored in large amounts, this led to the assumption that leptin is secreted through constitutive or regulated mechanisms. Leptin is also produced by other tissues including the fundus of the stomach, placenta, and mammary epithelium but in smaller amounts<sup>25,26</sup>. Yesim et al demonstrated that the concentration of leptin in breast milk correlated positively with serum leptin levels in the mother and negatively with serum insulin and thyroxine levels<sup>26</sup>. It is higher during the first 180 days of lactation in order to regulate energy metabolism in the neonate. In the stomach, the cells that produce leptin are located in the lower half of the gastric glands where it is secreted into the blood and gastric juices to acutely control satiety in combination with cholecystokinin (CCK) from the vagal nerve<sup>27</sup>. If leptin reaches the intestine, it can participate in food digestion and absorption, while the leptin receptors are located in the fundic and antral epithelial cells and most of these receptors are of the longest form, Ob-Rb, which has the longest intracellular domain<sup>27</sup>. In spite of the wide distribution of leptin production sites, adipose tissue is considered the main site for leptin synthesis since the correlation between blood leptin concentrations and the level of its mRNA in the adipose tissue is strong and

positive<sup>28,29</sup>. In addition, mRNA concentrations are increased in ventromedial hypothalamic lesions or with mutations in the receptor.

Leptin is secreted from the adipose tissue to maintain homeostatic control of adipose tissue mass in the body through regulating the activity of feeding centers in the hypothalamus to control food intake, energy expenditure and metabolism. When the fat mass in the body decreases, the plasma level of leptin will decrease leading to an increase in the appetite and decrease energy expenditure in the body and increasing fat mass. Similarly, leptin levels will increase when there is a decrease in fat mass and depress appetite and reduce body weight. In addition, adipocyte size determines leptin synthesis and secretion as large adipocytes contain more leptin than smaller ones<sup>30</sup>.

Therefore, leptin mRNA expression in adipose tissue and circulating leptin are increased in obese people and leptin is positively correlated with BMI and percent body fat<sup>30,31</sup>. In contrast, thin individuals and those showing progressive weight loss have low levels of leptin in the blood and low leptin mRNA expression in adipose tissue as their fat stores are low<sup>31</sup>. Considine et al showed that serum leptin levels in obese people were  $31.3 \pm 24.1$  ng/ml in comparison to  $7.5 \pm 9.3$  ng/ml in thin subjects<sup>32</sup>.

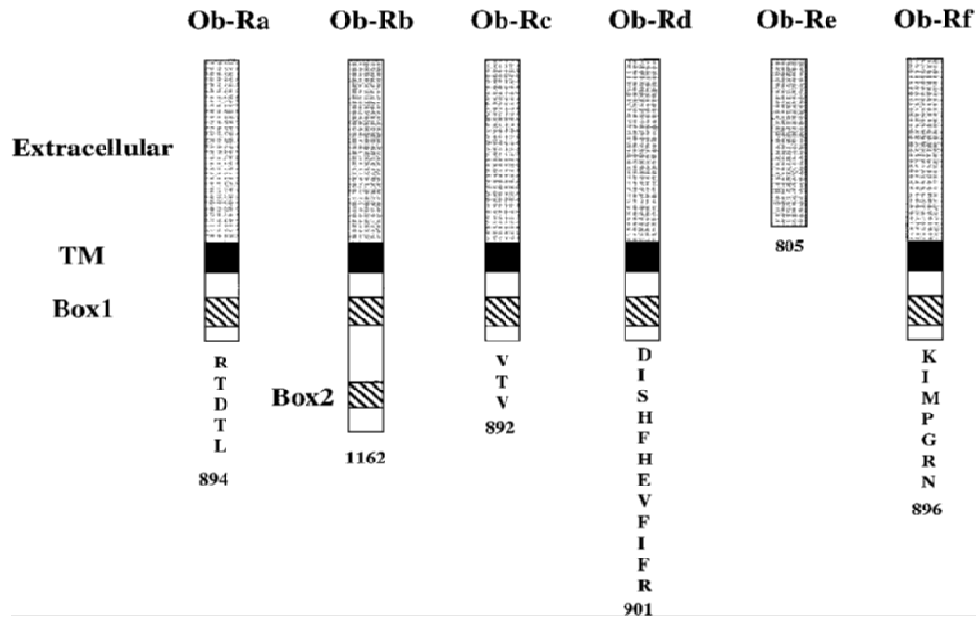
Although mutations of the ob gene in humans are rare, such mutations may cause hyperphagia, morbid obesity (in both humans and mice, its associated with normal birth weight followed by rapid development of obesity)<sup>33</sup>, and impair the hypothalamic hypogonadism axis<sup>34</sup> (primary amenorrhea in females; signs of delayed fertility in males such as no beard, scanty axillary and pubic hair, bilateral gynecomastia with small penis

and testes)<sup>33</sup>. Furthermore, insulin levels are elevated and insulin resistance is worsened with age in humans with leptin deficiency<sup>35</sup>.

### *Leptin Receptors (Ob-R)*

The leptin receptor is a member of the class I cytokine receptor family which includes the receptor for interleukin 6 (IL-6), leukemia inhibitory factor (LIF), and granulocyte-colony stimulating factor (GCSF). Baumann et al demonstrated that among the isoforms of leptin receptor Ob-Rb, the full length isoform can activate signaling pathways similar to that of the IL 6 inflammatory receptor<sup>36</sup>.

Using expression cloning, Tartaglia et al<sup>37</sup> identified and isolated the leptin receptor from the choroid plexus of a mouse. The leptin receptor gene is located on chromosome 17 and consists of 18 exons and 17 introns. The leptin receptor is 1162 amino acids<sup>38</sup> and present mainly in the hypothalamus and cerebellum but is also expressed in other tissues including the stomach, placenta, and vasculature<sup>39</sup>. Variations in mRNA splicing of the Ob-R produces 6 isoforms of the receptor (Ob-Ra, Ob-Rb, Ob-Rc, Ob-Rd, Ob-Re, Ob-Rf)<sup>40</sup>. These isoforms are similar in their extracellular ligand binding domain at the amino terminals, but they vary significantly in the intracellular structure and in signaling at the carboxy terminal<sup>41</sup>.



**Figure 3.** The various isoforms of the leptin receptor. The Ob-Rb isoform share extracellular binding domains, but vary in the intracellular motif. Ob-Rb is longest and Ob-Re lacks the transmembrane and intracellular motifs. Image from Ahima RS, Flier JS. *Leptin. Annu Rev Physiol.* 2000;62(1):413-437.

Ob-Ra is considered a leptin hormone transporter. Its structure is similar to Ob-Rb, except it lacks the cytoplasmic portion and therefore does not have signaling properties<sup>42</sup>. Ob-Rc is another short form of the receptor. Both Ob-Ra and Ob-Rc are expressed in significant concentrations in the choroid plexus and micro vessels suggesting that they act as blood-brain barrier transporters<sup>43</sup>. This occurs through transcytosis wherein leptin is transferred from blood to the cerebral spinal fluid (CSF), although concentrations are very low in CSF compared to the blood.

The Ob-Re receptor lacks the intracellular and trans membrane domains. Thus, it is considered a circulatory receptor. The half-life and clearance rate of leptin are determined by this soluble receptor. Therefore it is important in the regulation of leptin bioavailability and activity. Guoqing et al<sup>44</sup> found that Ob-Re not only acts as a reservoir



of leptin in the circulation, it also plays a general role in reducing fat accumulation and preventing weight gain by lowering food consumption and increasing basal metabolic rate (BMR). Moreover, they concluded that Ob-Re expression is inversely correlated to body weight and leptin concentrations in obese humans having high leptin concentrations in the blood while Ob-Re expression remains similar to that of non-obese people. This results in elevated free leptin especially in leptin resistant obese individuals<sup>42</sup>.

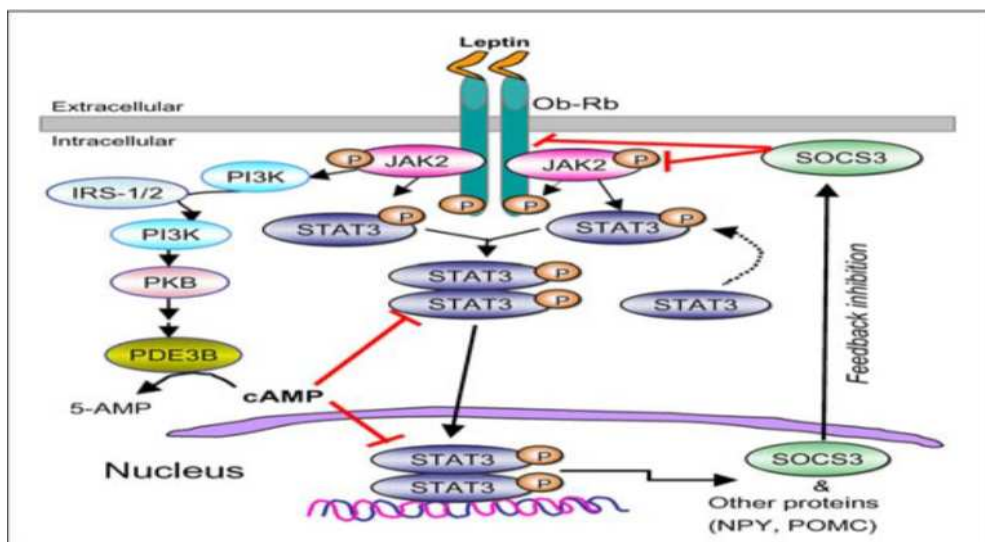
Ob-Rb receptor is considered the longest isoform and contains the intracellular domain important for the activation of signaling pathway that includes the enzymes JAK (Janus kinase) and STAT3 (signal transducers and activators of transcription)<sup>45</sup>. This receptor is highly expressed in the hypothalamic region that controls body weight indicative that leptin acts centrally on the hypothalamus to regulate body weight and energy expenditure. Central infusion of leptin into the cerebroventricular region has similar actions as endogenous leptin present in the peripheral circulation. Mutations in Ob-Rb are rare and have similar effects as mutations of the Ob gene including hyperphagia, obesity, and hypogonadism. In addition, mutations of the receptor gene may lead to impaired secretion of thyrotropin releasing hormone and growth hormone from the pituitary gland<sup>46</sup>. The signaling pathway through Ob-Rb involves leptin binding to the extracellular domain, leading to activation of JAK2 tyrosine kinase thereby enhancing tyrosine phosphorylation of specific sites on the leptin receptor and JAK2 to activate intracellular signaling.

The three major sites of phosphorylation are Tyr985, Tyr1077, and Tyr1138. Tyr1138 uses JAK2 to stimulate the phosphorylation of (STAT3) to promote transcriptional effects and signaling<sup>47</sup>. In contrast, Tyr985 activates Tyrosine-protein

phosphatase non-receptor type 11 (PTPN 11) instead and also binds to an inhibitor of Ob-Rb/JAK2 signaling called suppressor of cytokine signaling-3(SOCS3), and Tyr1077 uses STAT5<sup>47</sup>.

Several neurons in the hypothalamus are targets of leptin action through the neurotransmitters that they release including NPY (neuropeptide Y), POMC (pro-opiomelanocortin), galanin, and orexin. The STAT3 signaling pathway is present in these neurons<sup>48</sup> as well as other neurons that express leptin receptors. Defects in STAT3 signaling may interrupt the effect of leptin on POMC, but not NPY, suggesting that the inhibitory effect of leptin is controlled through different mechanisms<sup>49</sup>. The negative feedback control of STAT signaling is performed by SOCS proteins<sup>50</sup>, which contain src-homology domain (SH2) and c-terminal SOCS box<sup>51</sup>. Any defect in these mechanisms can lead to failure in the inhibitory mechanism of leptin signaling.

Another pathway involved in leptin signaling transduction is called PI3K-PDE3B-cAMP pathway. This pathway is found not only in the CNS<sup>52</sup>, but is also found in other tissues such as the insulin-producing pancreatic beta cells, hepatocytes, and adipocytes. In these cells, leptin activates an insulin-like pathway involving activation of PDE3B (PI3K dependent) and resultant reduction in intracellular cAMP<sup>53</sup>. The importance of cAMP in feeding and body weight regulation is supported by the evidence that when cAMP is injected centrally it causes increased feeding and induces NPY gene expression in the hypothalamus<sup>54</sup>. This pathway also interacts with the JAK2-STAT3 pathway as the PDE3 inhibition results in diminished leptin effects on STAT3<sup>55</sup>.



**Figure 4.** *Leptin intracellular signal transduction in the hypothalamus. Leptin binds to (Ob-Rb) and induces activation of Janus kinase (JAK), receptor dimerization, and JAK-mediated phosphorylation of the intracellular part of the receptor, followed by phosphorylation and activation of signal transducer and activators of transcription-3 (STAT3). Activated STAT3 dimerizes, translocates to the nucleus and transactivates target genes, including suppressor of cytokine signaling-3 (SOCS3), neuropeptide Y (NPY) and proopiomelanocortin (POMC). Image from Sahu A. Leptin signaling in the hypothalamus: Emphasis on energy homeostasis and leptin resistance. Front Neuroendocrinol. 2003;24(4):225-253.*

Leptin controls energy regulation and feeding through the hypothalamus, which is considered the primary center of feeding regulation<sup>57</sup>. The binding sites of leptin include the arcuate, ventromedial and dorsomedial hypothalamic nuclei and neuronal circuits in the brainstem<sup>58</sup>. Pellemounter et al<sup>59</sup> demonstrated that the injection of leptin into these sites inhibits food intake to a greater degree than administering leptin peripherally. Steven et al<sup>56</sup> showed that administration of exogenous leptin (recombinant human leptin injected subcutaneously) induces weight loss in lean individuals and in obese individuals,

even those with high endogenous leptin (indicating leptin resistance). Moreover, the weight loss was dose dependent with most of the loss occurring in adipose tissue.

James et al<sup>60</sup> showed that the release of leptin from adipocytes relies on activation of calcium-dependent channels that are influenced by insulin and glucose levels.

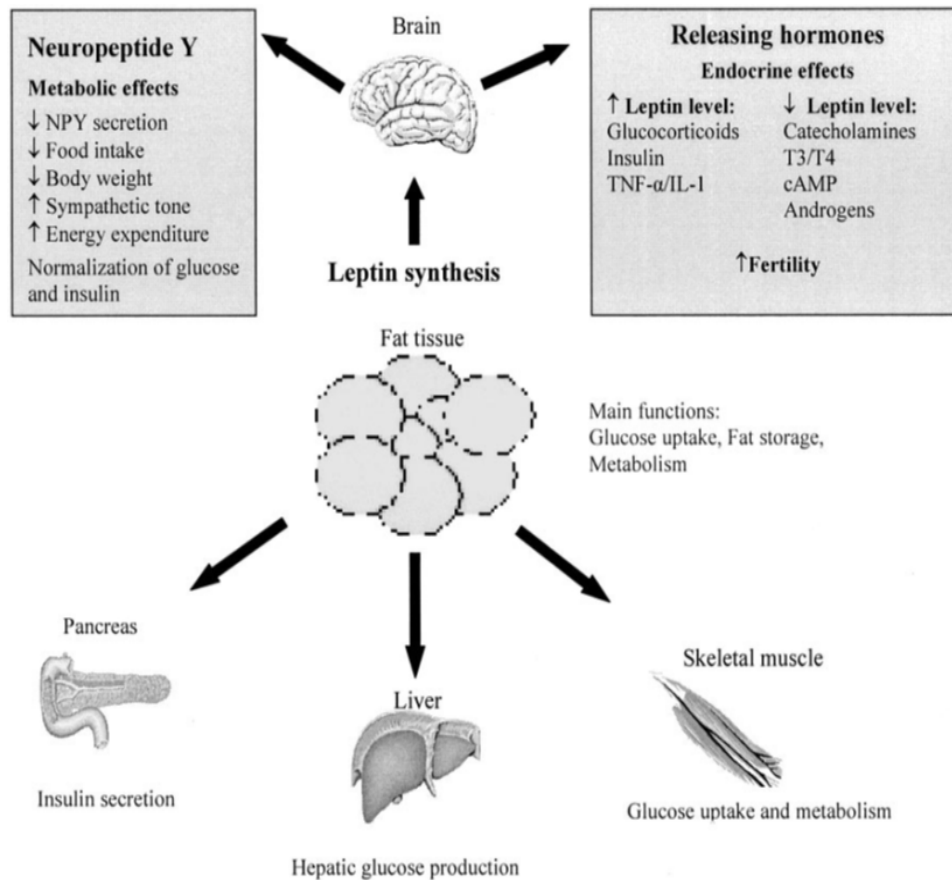
Circulating leptin is present in a free form or bound to plasma proteins (soluble receptor)<sup>61</sup>. The relative amount of leptin bound to plasma proteins tends to be higher in lean persons in comparison to obese individuals, which affects the bioavailability of leptin<sup>28,62</sup>.

After leptin crosses the blood-brain barrier, it influences the activity of various orexigenic and anorexigenic neurons located in different regions of the hypothalamus to produce different neurotransmitter or neuropeptides. Leptin suppresses orexigenic neuropeptides including neuropeptide Y (NPY), melanin concentrating hormone (MCH), agouti-related protein (AgRP), galanin, orexin, and galanin like peptide (GALP). In contrast, leptin stimulates the anorexigenic neuropeptides such as proopiomelanocortin (POMC; a precursor of  $\alpha$ -MSH that works through the MC4 receptor), cocaine and amphetamine regulated transcript (CART), neurotensin, and corticotropin releasing hormone (CRH)<sup>57,63</sup>. The leptin receptor Ob-Rb is co-expressed with NPY, AgRP, POMC, and CART in the arcuate hypothalamus, NPY and AgRP are expressed in the medial arcuate nucleus, while POMC and CART are expressed in the lateral arcuate nucleus<sup>64</sup>.

In addition to the effect of these neuropeptides on energy regulation and feeding behavior, these orexigenic and anorexigenic neuropeptides react with each other<sup>65,66</sup> as

the  $\alpha$ -MSH does not inhibit the effect of NPY but inhibits MCH, while a melanocortin agonist inhibits NPY<sup>67</sup>, and GLP-1 (glucagon like peptide-1) inhibits both MCH and NPY<sup>68</sup>.

NPY, which is produced by neurons in the arcuate nucleus, is considered a strong appetite stimulator and is involved in the regulation of several pituitary hormones. For example, it suppresses growth hormone through the stimulation of somatostatin and suppression of gonadotropins, in addition to regulating the pituitary adrenal axis<sup>69</sup> and participating in cardiovascular physiology and mood modulation. But in spite of its strong potential effect on feeding, NPY deficiency or lack of NPY Y1 receptors (involved in contraction of vascular smooth muscle cells) or NPY Y5 receptors does not result in gross reduction of feeding behavior or prevent mild late weight gain nor does it impair the response to leptin<sup>70-72</sup>. In contrast, increased NPY in the brain leads to increased obesity gene expression in white adipose tissue through stimulation of hyperinsulinemia<sup>73</sup>.



**Figure 5.** Action of leptin on the hypothalamus and peripheral organs (pancreas, liver, and skeletal muscle, Leptin produced by fat tissue will act on the hypothalamus by decreasing NPY which causes a reduction in appetite and body weight and increases energy expenditure, it also improves  $\beta$  cell function in the pancreas and glucose metabolism. Image from Meier U, Gressner AM. Endocrine regulation of energy metabolism: Review of pathobiochemical and clinical chemical aspects of leptin, ghrelin, adiponectin, and resistin. *Clin Chem.* 2004;50(9):1511-1525.

Masako et al showed that melano-concentrating hormone (MCH) deficiency results in hypophagia and weight loss<sup>74</sup>. The resultant decrease in leptin concentrations should cause hyperphagia, but rather hypophagia was observed. This indicates that MCH is important in the stimulation of hyperphagia associated with leptin deficiency. In the

same experiments, Masako concluded that the expression of POMC in the arcuate nucleus is reduced as a result of the reduced leptin concentrations, although the MCH deficiency in lateral hypothalamic neurons can cause reduced POMC expression in the arcuate nucleus.

MCH is a short-term appetite stimulant that does not activate sustained increases in food intake and weight gain. The axons of MCH neurons project to the cortex and tractus solitaries, parabrachial nucleus in hindbrain and are upregulated by fasting. In contrast, NPY plays a role in the regulation of food intake and causes acute hyperphagia and chronic weight gain with axons that project to the paraventricular and dorsomedial hypothalamic nuclei. NPY is up regulated by fasting and negatively affected by leptin and insulin<sup>75</sup> and both NPY and MCH gene mutations in rats leads to normal feeding behaviors<sup>21</sup> indicating they are not essential for normal eating.

Katsunori et al found that mutation of serotonin 5-HT<sub>2C</sub> receptors in the lateral, arcuate, dorsomedial hypothalamus, and the paraventricular nuclei leads to the development of hyperphagia, late onset obesity and disturbance of leptin signaling<sup>76</sup>. Responses to external leptin administration were normal indicating that the action of serotonin receptors does not require leptin.

Other neurotransmitters and peptides involved in the mechanism of leptin signaling in the hypothalamus include glucagon like peptide-1 (GLP-1), urocortin, bombesin, and serotonin<sup>12</sup>. As mentioned, the orexigenic hormones that stimulate appetite through the arcuate nucleus are suppressed by leptin. However, both orexin and leptin interact centrally leading to increased heart rate and arterial pressure (especially

during stress) suggesting that they may act as a chemical mediator of hypertension associated with obesity<sup>77</sup>. Leptin interact with other neurotransmitters/neuropeptides centrally to regulate feeding, reproduction, immune and inflammatory response, hematopoiesis, angiogenesis, bone formation and wound healing through a complex network through the hypothalamus and other parts of the brain<sup>78</sup>. An example is the NPY/AgRP and POMC/CART neurons that project to the paraventricular nucleus to mediate the effect of leptin on the neuro-endocrine axis and autonomic function<sup>79</sup>.

#### *Leptin and Bone Metabolism*

Dietary weight loss takes place in both obese and lean people and is associated with decreased bone mass and changes in markers of bone formation<sup>80</sup>. Leptin plays a role in the altered bone metabolism<sup>81</sup>. Centrally, leptin regulates the expression of RANKL (receptor activator of nuclear factor kappa-B ligand). Through increased expression of CART, RANKL affects bone resorption through activation of osteoclasts. Leptin also acts on osteoblasts through the central activation of the sympathetic nervous system<sup>82</sup>. In the periphery, the effects of leptin's effects include osteoblast activation, cell proliferation, collagen synthesis, and mineralization, although other factors may be involved such as variation in diets, level of physical activity and insulin sensitivity<sup>82</sup>.

#### *Leptin and Thyroid Hormone*

The thyroid hormones (T3 and T4) are secreted in response to stimulation of TSH released from the anterior pituitary gland. The concentrations of thyroid hormones are regulated by feedback mechanisms between the thyroid and the pituitary, which is under the control of the hypothalamus (paraventricular nucleus). The hypothalamus secretes



thyroid releasing hormone (TRH), which in turn, stimulates the release of TSH from the anterior pituitary. In fasting, the level of T3 and T4 are decreased in response to low TSH as a result of suppression of TRH. Legardi et al found that leptin administration during fasting normalizes thyroid hormone concentrations as a result of leptin-mediated prevention of reduced TRH in the hypothalamus<sup>83</sup>.

### *Leptin and Regulation of Immunity*

Leptin increases the production of certain cytokines from the T cells like IL-2 and interferon- $\gamma$  (INF-Y) while it decreases IL-4 and plays an important role in the regulation of T helper balance Th1/Th2 as it increases the production of cytokines from macrophages<sup>84</sup> and enhances the alloproliferative response of human peripheral blood lymphocytes<sup>85</sup>. Treatment with leptin also reduces apoptosis in the thymus<sup>85</sup>. Moreover, leptin deficiency in mice diminishes hematopoiesis and delays dermal wound healing while administration of leptin accelerates wound healing<sup>86,87</sup>.

In the case of starvation, leptin concentrations decline with the reduction in adiposity which is accompanied by a decline in immune function to conserve energy expenditure for more important systems like the CNS and metabolism, a situation reversed by leptin administration<sup>88</sup>. Patients with lipodystrophy (chronic disease of fat tissue) have significantly reduced plasma leptin concentrations. Hamilton et al demonstrated another role of adipose tissue in the regulation of leptin secretion, where it was shown that the increased size of adipocytes affects the rate of Ob mRNA transcription leading to more leptin in the blood<sup>30</sup>. Serum leptin concentrations vary directly with percentage of body fat and there is a significant positive correlation between

BMI and leptin concentration<sup>89,90</sup>. Although serum and adipose tissue leptin concentrations are both increased in obese patients, these patients are leptin-resistant<sup>89</sup>.

Regarding food intake, leptin concentration does not change acutely following meals. Rather it requires chronic overfeeding to increase concentrations. Similarly, it takes hours from the start of a fast to measure decreases in leptin concentrations. Therefore, leptin cannot be considered a mediator of immediate post-meal satiety<sup>91,92</sup>. This is a possible explanation for the mild increase in leptin during short-term fasting because the body still has adequate fat stores that can be used to meet energy requirements<sup>93</sup>.

Leptin exhibits a diurnal rhythm wherein the concentration is higher during the night compared to the day. This probably occurs to suppress appetite during sleep and increase activity and energy expenditure during the day<sup>94</sup>. Schoeller et al<sup>95</sup> showed that reversing day/night patterns of subjects resulted in a similar reversal in the peak concentrations of leptin. Moreover, shifting meal times was accompanied by shifted leptin concentrations where peak levels were associated with the synthesis of cholesterol. Another study showed that leptin concentrations at 2:00 pm are 30-100% higher than serum leptin concentrations in the morning and early afternoon<sup>94</sup>.

Insulin is considered an important long-term regulator of leptin appearance. Increased insulin concentrations after a meal are associated with increased leptin mRNA concentrations about 6 hours later that were found to be in response to insulin as opposed to the meal<sup>96</sup>. Regarding long-term regulation, Korbonits et al found that six months after surgical removal of an insulinoma, the level of insulin declined and led to decreased BMI

and leptin in one subject<sup>92</sup>. Considine et al concluded that the role of insulin in the regulation of leptin concentrations depends on previous nutritional status<sup>89</sup>. Data from Mueller et al shows that leptin secretion is not stimulated directly by insulin but instead by the effects of insulin on glucose delivery and metabolism in the adipocytes<sup>90</sup>. Therefore, the quantity of glucose metabolized by adipocytes will lead to larger leptin responses.

Glucocorticoids increase the expression of leptin mRNA in adipose tissue resulting in increased circulating leptin, while increased intracellular cAMP decreases the expression<sup>97,98</sup>. Although glucocorticosteroids also stimulate the secretion of NPY from the hypothalamus, its effect is suppressed by leptin<sup>99</sup>. The effect of different glucocorticoids on leptin is variable. While dexamethasone and hydrocortisone increase leptin levels about 3-4 fold, other steroids like aldosterone are ineffective<sup>100</sup>.

Thyroid hormone is negatively associated with serum leptin concentrations whereas thyroid stimulating hormone is positively correlated with leptin<sup>101</sup>. Hector et al showed that administration of thyroid hormone lowers serum leptin concentrations<sup>102</sup>. A possible explanation for this relationship is that as thyroid hormone increases, leptin declines to reduce the catabolic state, since both hormones increase metabolic rate<sup>101</sup>.

Grunfeld et al showed that cytokines and endotoxins during infections may induce the secretion of leptin and its mRNA from adipose tissues<sup>103</sup>. Inflammatory factors like TNF $\alpha$  and interleukin-1 (IL-1) increase serum and adipose leptin levels and therefore may induce anorexia during infections<sup>103</sup>. Bornstein et al concluded that leptin also acts

as a stress-related hormone that may play a role in survival as a part of the host defense mechanism<sup>104</sup>. Therefore, decreased leptin might affect the ability of fight infections.

Matsuoka et al examined the magnitude of changes in serum leptin and body fat mass in relation to growth hormone secretion and found that growth hormone does not directly effect leptin levels<sup>105</sup>. Rather, a reduction in body fat mass causes reduced leptin concentrations. In turn, leptin acts as a messenger to regulate growth hormone secretion.

### *Leptin resistance*

Hyperleptinemia is defined as a state of elevated circulating leptin concentrations in response to increased caloric intake. This elevated state results in resistance to the catabolic activity, decrease feeding behavior and prevention of increased body weight and obesity in response to leptin (i.e., leptin resistance). Obesity has been associated with leptin resistance as well as insulin resistance, especially in type 2 diabetes<sup>106</sup>, as food intake promotes increased adiposity leading to cellular leptin resistance. However, leptin resistance can be found in normal weight rats as well<sup>107</sup>. In fact, Wang et al<sup>107</sup> demonstrated that induction of overfeeding in normal weight rats caused insulin and leptin resistance after just 3 days. This form of resistance is usually detected in the hypothalamus. Dominique et al<sup>108</sup> further showed that high fat diets result in defective leptin signaling to the hypothalamus as well as defects in activating the STAT3 pathway. Obesity may further contribute to central leptin resistance by causing endoplasmic reticulum stress thereby affecting the ObRb<sup>109</sup>.

Leptin resistance can be found in cases of genetic mutations that cause deficiency in the production of leptin or its receptors, and can also be caused by defects in leptin

signaling. In the case of mutations of the gene responsible for leptin receptor formation, it was found that patients with a defect in this gene have high levels of leptin receptors that are not attached to the surface of target cells due to a lack of the trans membrane and intracellular domains. These free receptors circulate in the blood where they attach to leptin produced in the body thereby preventing the normal effects of leptin<sup>61</sup>. Defects in leptin signaling can be attributed to several factors:

1) Saturable transport of leptin across the blood brain barrier<sup>110</sup>

This is supported by evidence showing the ratio between leptin in cerebral spinal fluid and plasma is lower in obese patients<sup>111</sup> and the central administration of leptin in obese rats decreases food intake while peripheral administration does not cause the same effect<sup>112</sup>. This usually occurs with the development of obesity.

2) Defect in leptin signaling in the neural cells

During a state of hyperleptinemia, the raised leptin concentrations stimulate the phosphorylation of Tyr985 in the ObRb limiting the signaling in this receptor. Moreover, hyperleptinemia increases SOCS3, which blunts the receptor signaling<sup>113</sup>. In addition, decreased STAT3 phosphorylation contributes to defective leptin signaling<sup>114,115</sup>.

3) Defect in the neural pathway participating in leptin function

Disruption of leptin signaling through the melanocortin pathway, which normally functions to reduce food intake, may be caused by various genetic mutations in the melanocortin peptide or its receptor, pharmacological agents, as well as diseases or lesions in the hypothalamus<sup>116,117</sup>.

- 4) Increased adiposity and hyperleptinemia as a result of defects in tissues not related to leptin signaling pathways

These defects may include mitochondrial uncoupling that promotes leakage of electrons, thereby causing oxidative stress and preventing the transformation of fat energy into heat<sup>118</sup>. Leptin resistance can also be a consequence of an ionic imbalance of zinc, calcium, and magnesium in food resulting in oxidative stress and eventually leptin resistance, a situation that can be restored by antioxidant administration<sup>119</sup>. Moreover, Montague et al showed that deletion of a guanine nucleotide in codon 133 of the gene for leptin resulted in morbid obesity associated with low leptin concentrations in humans<sup>120</sup>.

Besides the presence in the CNS, leptin receptors (OB-Rb) are also found in aortic, umbilical, adipose and dermal micro vascular endothelial cells<sup>121</sup>, and the activation of these receptors causes tyrosine phosphorylation of the STAT3 pathway within these cells. Activation of this pathway promotes angiogenesis especially in the adipose tissue where it increases lipid release and oxidation as a part of the peripheral actions of leptin in the maintenance of energy homeostasis<sup>122</sup>.

Regarding leptin and atherosclerosis, Taleb et al demonstrated that atherosclerotic lesions in leptin-deficient mice are smaller in size compared to those without leptin deficiency<sup>123</sup>. They also documented a significant correlation between serum leptin levels and cardiovascular disease, especially atherosclerosis, which is carried through the immune pathway, as leptin induces the proliferation of immune and inflammatory cells in many diseases like colitis, diabetes, and atherosclerosis.

Leptin also increases sympathetic outflow when administered intravenously or intracerebroventricularly causing increased blood pressure and heart rate<sup>124</sup>. In contrast, leptin-deficient obese rats have low blood pressure readings in comparison to normal rats and develop increased blood pressure after peripheral leptin administration<sup>125</sup>. This is attributed to the effects of leptin on renal sympathetic nerve activity even in the presence of central resistance to leptin for weight loss<sup>126</sup>.

Leptin induces coronary artery vasodilation and improves coronary blood flow up to 40% in some instances. This mechanism of vasodilation is endothelium-independent and does not occur through the NO mechanism<sup>127</sup>. However, this vasodilator effect requires high doses of leptin that are above levels found in morbidly obese people<sup>128</sup>, while hyperleptinemia in obese people can negatively affect myocardial function and lead to heart failure<sup>129</sup>.

Hyperleptinemia that presents in obesity and metabolic syndrome can cause oxidative stress and endothelial dysfunction, which leads to atherosclerosis<sup>130</sup>. In fact, there is a strong correlation of serum leptin concentrations with acute phase reactants markers like C reactive protein<sup>131</sup> and evidence shows any defect in leptin signaling can work as a protector against atherosclerosis<sup>132</sup>.

### *Leptin and diabetes*

Exogenous administration of leptin in patients with type 1 diabetes mellitus corrected insulin deficiency and improved glucose and lipid profile although insulin therapy was not discontinued<sup>133</sup>. As most patients with type 2 diabetes mellitus are obese, leptin concentrations in those patients are high and concentrations in women are higher

than men. This is attributed to women having a higher fat percentage<sup>134</sup>. Therefore, type 2 diabetic patients show a refractory response to the metabolic effect of leptin administration. However, the anti-diabetic benefits of leptin can be seen in thin patients with normal leptin concentrations<sup>135</sup>. On the other hand, diabetes does not seem to influence acute and chronic leptin concentrations. Rather, what raises the leptin concentration is the state of hyperinsulinemia in experimental techniques and after long durations<sup>136</sup>, which could be a result of the atrophic effect of insulin on adipocytes.

As leptin receptors are found on  $\beta$  cells in the pancreas, leptin can inhibit insulin production through the activation of ATP-sensitive K<sup>+</sup> channels. This suppression of insulin by hyperleptinemia is associated with increased insulin insensitivity and can explain metabolic syndrome associated with insulin resistance<sup>137</sup>. In addition, leptin administration to leptin-deficient mice improves skeletal muscle sensitivity to insulin<sup>138</sup>, which is considered the predominant target of insulin-mediated glucose disposal. Leptin also induces insulin-dependent suppression of hepatic glucose output through modulating hepatic gluconeogenesis enzymes<sup>139</sup>. These effects of leptin on glucose homeostasis and insulin resistance in obese people occurs centrally through the proopiomelanocortin (POMC) neurons in the arcuate nucleus of the hypothalamus<sup>140</sup>. In certain other clinical diseases like lipodystrophy, a disorder of adipose tissue homeostasis, leptin administration also corrects hyperglycemia and insulin resistance that accompany the disease<sup>141</sup>.



## **Nitric Oxide and Endothelial Dysfunction**

### *Inflammation*

Inflammation is the response of the immune system in living tissues to local injury or microscopic invaders. It leads to local accumulation of blood cells and fluids in order to isolate the causative microorganism and repair the injured area. The cells and the extracellular material in the injured area will release signals (chemical and physical), which initiate inflammatory reactions<sup>142</sup>. The chemical signals act on the microcirculation to increase fluid and white blood cell (leukocytes, lymphocytes, and plasma cells) exudates.

In acute inflammation, two sets of vascular events occur. First, changes in vascular flow and caliber occur to control the amount of exudates at the inflammation site. Chemical mediators released at the site of inflammation cause the blood vessels to dilate and the venules to contract<sup>143</sup>. These events promote increased blood flow to the site of injury. Secondly, vascular permeability becomes altered. Increased leakage of particles and fluids through the vascular membrane leads to more protein loss, while larger particles like lipoprotein are retained<sup>144</sup>. The endothelial lining of micro-vessels act as an active barrier responsible for maintaining circulatory homeostasis by regulating the exchange between blood and tissues. This passage of protein and fluid through endothelial cell-cell junctions is also important in vascular inflammation as fluids and inflammatory cells pass through the endothelial membrane to the infected or inflamed tissue. This process is mediated by increased immune cells and inflammatory mediators

like histamine, thrombin, vascular endothelial growth factor, neutrophil and neutrophil related cytokines like interleukins (ILs) and tumor necrosis factor (TNF)<sup>145</sup>.

In general, the behavior of any living tissue in acute inflammation is controlled by both functional and structural events or changes that take place in the microcirculation. These changes include: impaired vasomotor function, recruitment of leukocytes, diminished endothelial barrier function<sup>145</sup>, angiogenesis, and increased thrombosis, which is characterized by increased tissue plasminogen activator inhibitor and decreased C anticoagulant pathways to inhibit fibrinolysis<sup>146</sup>. This inflammatory response is not associated with acute injuries, but also accompanies many pathological conditions like obesity, cancer, diabetes mellitus, and hypertension. Among these pathological conditions, atherosclerosis represents an example of chronic inflammation of blood vessels which can cause endothelial dysfunction<sup>147,148</sup>.

### *Endothelial Dysfunction*

The impairment of physiologic endothelium dependent relaxation may occur in hypertension, diabetes, hypercholesterolemia, and atherosclerosis<sup>149</sup>. Endothelial dysfunction broadly occurs when the physiologic and protective properties of the vascular endothelium are lost or damaged such as the damage that occurs during percutaneous procedures in coronary arteries. Alternatively, endothelial dysfunction can be a result of oxidative stress, hyperglycemia, advanced glycation end products, metabolic responses to free fatty acids, and inflammatory cytokines<sup>150-152</sup>. Endothelial dysfunction can be judged by assessing the vasodilator response. Two main vascular alterations that accompany micro vascular dysfunction: these alterations are the

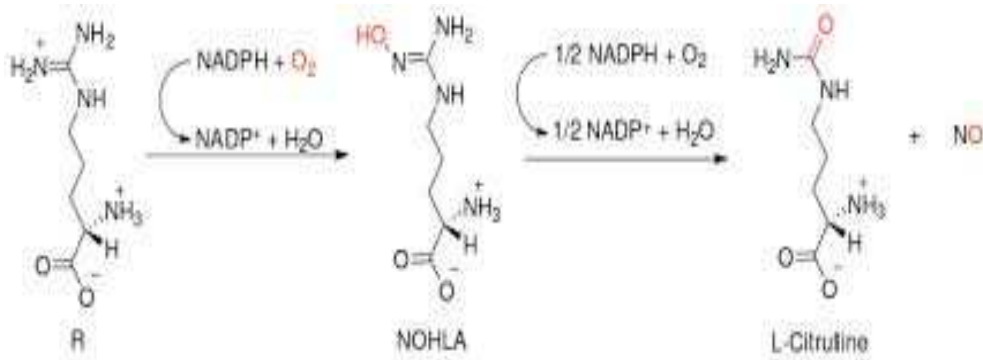
generation of reactive oxygen species (ROS)<sup>153</sup> and decreased availability of the endogenous vasodilator nitric oxide (NO)<sup>154</sup>.

### *Nitric oxide (NO)*

Nitric oxide is a free radical gas molecule produced from the conversion of L-arginine to L-citrulline by endothelial nitric oxide synthase (eNOS) in the endothelial cells of blood vessels (as described below)<sup>155</sup>. It is an uncharged molecule that is highly soluble in hydrophobic media, which is what enables it to diffuse easily through cell membranes. NO takes part in the vascular homeostasis and in the regulation of vasodilation and vasoconstriction through the increase of cGMP. It was discovered in 1980 by Furchgott and Zawadiskii as an endothelial-derived relaxing factor<sup>154</sup>. In normal conditions, the endothelial cells produce NO in small amounts to maintain vascular tone. But the rate of synthesis is increased in response to chemical stimuli, such as thrombin, ADP, serotonin, acetylcholine, and bradykinin, or physical stimuli like shear stress and cyclic strain<sup>156</sup>.

NO is produced by nitric oxide synthase enzymes, which are hemoprotein enzymes that catalyze a five step electron oxidation of the terminal guanidine nitrogen atom of L-arginine to L-citrulline and NO<sup>157</sup>. NOS is composed of a N-terminal oxygenase domain and a C-terminal reductase domain with a calmodulin recognition sequence between them<sup>158</sup>. Activity of the enzyme requires the presence of flavin mononucleotide and flavin adenine dinucleotide, tetrahydrobiopterin (BH4), calcium, and heme<sup>159</sup>. As the calcium concentration inside endothelial cells increases, it affects the

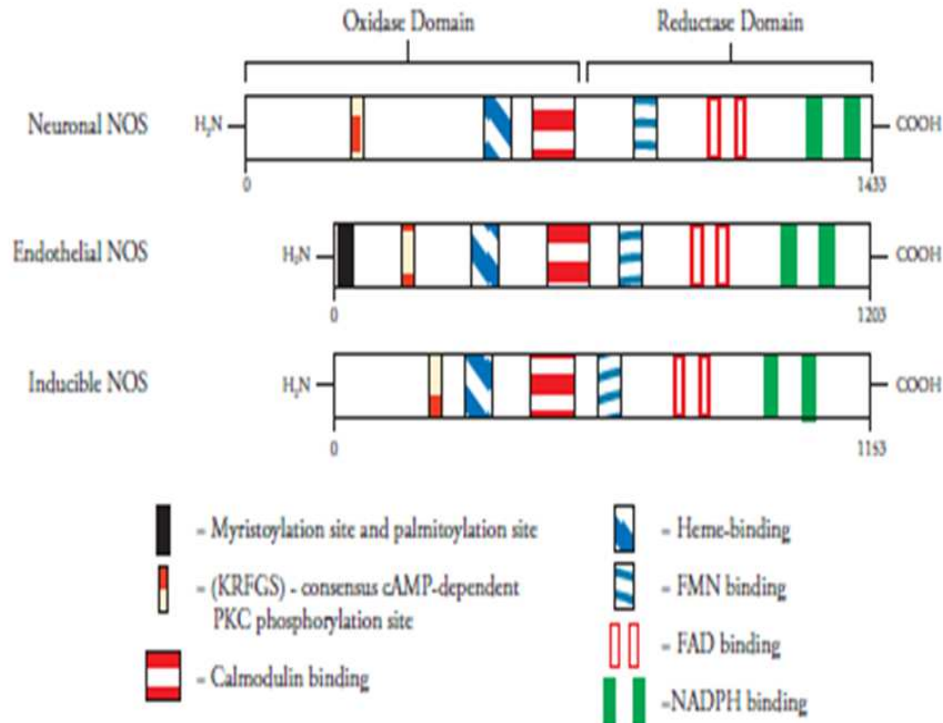
ability of calmodulin to react with the transfer of electrons from NADPH to the heme iron thereby acting as a catalyst in the synthesis of NO<sup>160</sup>.



**Figure 6.** The synthesis of NO by nitric oxide synthase. Image from [http://en.wikipedia.org/wiki/Nitric\\_oxide\\_synthase](http://en.wikipedia.org/wiki/Nitric_oxide_synthase)

NOS are also regulated by NO which can act as a negative feedback inhibitor of the enzyme activity as the NO molecule reacts with the ferrous hemoprotein part of the enzyme to temporarily inactivate NOS<sup>161</sup>. Other factors that influence the activity of these enzymes include BH<sub>4</sub> deficiency or L-arginine deficiency. In fact, the administration of L-arginine improves the enzyme function in rabbit thoracic aorta and, as a result, the availability of NO<sup>162</sup>.

In contrast, methylation of arginine by methyltransferase in healthy individuals results in asymmetric dimethyl arginine, which acts as an inhibitor of the enzyme<sup>163</sup> as well as a vasoconstrictor that induces hypertension. Marked elevations in asymmetric dimethyl arginine levels are seen in certain diseases like hypertension, hypercholesterolemia, stroke, and diabetes mellitus<sup>164,165,166</sup>.



**Figure 7.** Nitric oxide synthase isoforms. Image from <https://www.caymanchem.com/pdfs/780001.pdf>

There are 3 isoforms of the nitric oxide synthase enzyme: neuronal (nNOS), endothelial (eNOS), and inducible (iNOS)<sup>167</sup>. The nNOS isoform is found mainly in the nervous system and produces NO in scanty amounts during normal physiological conditions. It plays a role in the process of memory<sup>168</sup>, feeding behaviors<sup>169</sup>, and communication in both peripheral and central neurons<sup>170</sup>. Inducible NOS produces NO as a response to microbial endotoxins as well as cytokines and the quantity of NO produced by iNOS are usually large<sup>167</sup>. NO produced by iNOS acts as a vasodilator to resist vasoconstriction that accompanies certain pathological conditions like septic shock<sup>167</sup>.

Increased production of superoxide (O<sub>2</sub><sup>-</sup>) during inflammatory stress and high fat intake stimulates iNOS protein synthesis and this, in turn, causes more O<sub>2</sub><sup>-</sup> production.

Superoxide is a scavenger of NO in the blood vessel wall resulting in the formation of peroxynitrite (ONOO-) causing impaired vasodilation<sup>171</sup>. Deficiency of eNOS in experiments conducted on mice is associated with clinical complications like atherosclerosis and hypertension<sup>172</sup>, vascular smooth muscle cell proliferation during injury<sup>173</sup>, hypercoagulability<sup>174</sup>, and increased diet-induced atherosclerosis<sup>175</sup>. eNOS and nNOS are found in BAT (brown adipose tissue) and because NO inhibits cytochrome oxidase, NO participates in energy regulation of BAT through decreased mitochondrial respiration<sup>176</sup>. Ablation of iNOS in leptin-deficient mice leads to increased thermogenesis, body temperature and energy expenditure<sup>176</sup> whereas abnormalities in eNOS may be associated with obesity and metabolic changes in diabetes.

eNOS usually is activated by phosphorylation of the serine and threonine residues in the amino acid sequence of the enzyme<sup>177</sup> by mechanical mediators like shear stress, or chemical mediators like acetylcholine, insulin and estrogen<sup>178</sup>. leading to increase NO production. Importantly, eNOS production of NO is decreased in diabetes, hypertension, metabolic syndrome, obesity, and in response to inflammatory factors like IL-6 and TNF  $\alpha$ <sup>179,180</sup>. Elevated NO concentrations negatively feedback to inhibit NOS and oxidized LDL limits NO activity<sup>181</sup>, while administration of L-arginine improves endothelial function in animals fed a high cholesterol diet. Oral administration of L-arginine in patients with heart failure showed improvement in forearm blood flow and increased distances during a 6-minute walk<sup>182</sup>. The administration of statins, which are 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, is used as first-line mechanism to prevent cardiovascular disease associated with atherosclerosis by reducing serum cholesterol. The administration of statins is associated with improvement of endothelial

function by removing oxidative stress and increasing NO bioavailability through increased eNOS activity, inhibition of eNOS uncoupling and reduced production of ROS from NADPH oxidase<sup>183,184</sup>. Sweazea et al<sup>185</sup> showed that high fat and high sucrose diets in rats may cause oxidative stress and impaired vasodilation, although the mechanism is different between the two diets wherein high fat diet rats develop vascular dysfunction through scavenging of NO by O<sub>2</sub><sup>-</sup>, while high sucrose diet rats develop dysfunction through overproduction of H<sub>2</sub>O<sub>2</sub>. Impaired vasodilation is also attributed to decreased vascular smooth muscle tone through decreases in calcium stores or reduced smooth muscle sensitivity to calcium<sup>186</sup>.

During hyperbaric oxygenation, eNOS regulates cerebral blood flow to protect the brain against ROS-induced vasoconstriction<sup>187</sup>. While apoE/eNOS knock-out mice show evidence of ischemic heart disease, left ventricular dysfunction, and aortic aneurysm, similar to what happens in atherosclerotic and cardiovascular diseases<sup>175</sup>. In addition, eNOS knockout mice are hypertensive despite the presence of other counter regulatory mechanisms like cardiac, neuronal, and hormonal<sup>188</sup>.

#### *Function of nitric oxide*

NO has an important role in regulating vascular tone and blood flow as NO produced in the endothelium diffuses to smooth muscle cells causing them to relax, leading to vasodilation and lowering blood pressure<sup>190</sup>.

#### *Vascular endothelium and blood vessels contraction*

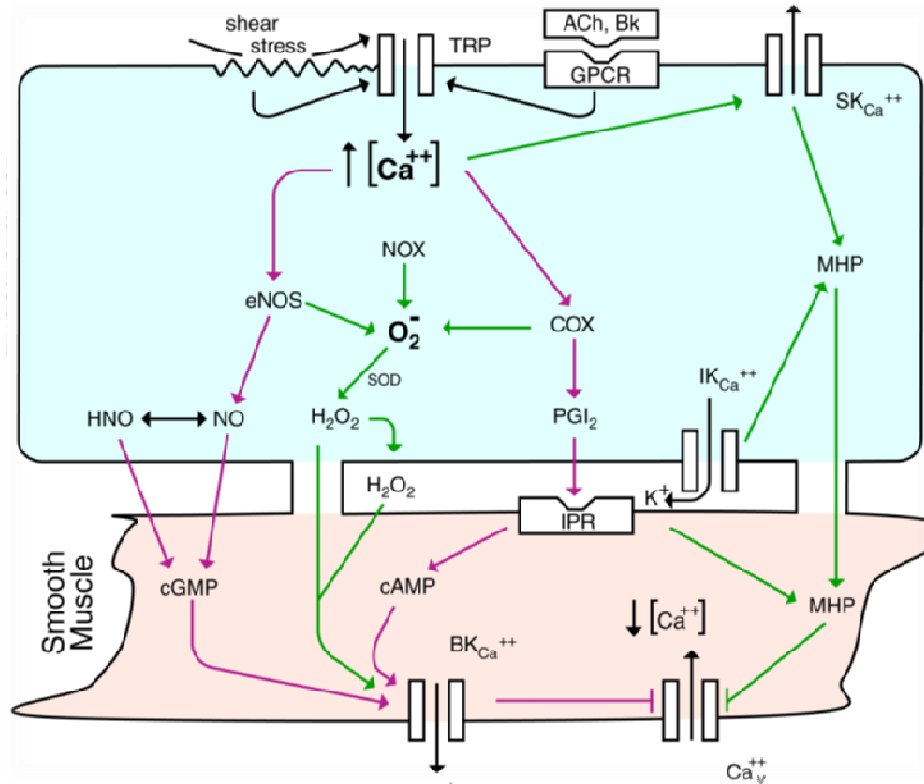
The endothelial cells lining the lumen of blood vessels participate in the adjacent smooth muscle contractions<sup>189</sup> and lead to the relaxation of arteries and veins through

stimulation of muscarinic receptors via the release of endothelial-derived relaxing factors (EDRFs). Acetylcholine binding to the muscarinic receptors may also activate the release of EDRFs<sup>190</sup>. This type of relaxation is more prevalent in arteries than veins, but it can be found in some large veins such as the pulmonary vein<sup>191</sup>. Absence of the endothelial lining prevents the vasodilator response to acetylcholine and instead causes contraction when large amounts of acetylcholine are administered<sup>192</sup>. Vasorelaxation induced by ATP, ADP, and increases in extracellular calcium are endothelium-dependent<sup>193</sup>. The endothelium can also be a source of vasoconstrictor signals where it can augment the contractions started by norepinephrine and prevent vasodilation by inhibition of cyclooxygenase and thromboxane synthase<sup>194,195</sup>.

Vasomotor reactivity of blood vessels is regulated by the close proximity of vascular smooth muscle cells and the adjacent endothelial cells<sup>192,196</sup>. Histological and physiological analyses show that the endothelial cell lining of blood vessels are separated from the smooth muscle cells by the internal elastic lamina<sup>197</sup>. Myoendothelial gap junctions (MEGJs) physically connect endothelial cells and smooth muscle cells<sup>198</sup>. Activation of endothelial cells by shear stress or activation of GPCR ligands (G protein coupled receptors) by acetylcholine<sup>198</sup>, increases calcium influx through receptor-operated channels (ROC3) and store-operated channels (SOC3)<sup>199</sup>. As the concentration of intracellular calcium increases, two pathways become activated: EDHFs (endothelial-derived hyperpolarizing factors) and EDRFs (endothelial-derived relaxing factor)<sup>190,200</sup>. EDRFs include NO, which diffuses through myoendothelial gap junctions to the smooth muscle cells to activate cGMP, as well as cyclooxygenase that produces prostaglandins, which react with a receptor on the smooth muscle surface called IRP to activate the



release of cAMP<sup>201</sup>. Activated cAMP and cGMP, in turn, activate calcium-dependent potassium channels leading to the inhibition of voltage-gated calcium channels resulting in decreased calcium concentrations in smooth muscle cells causing relaxation<sup>201</sup>. Increased concentrations of intracellular calcium in endothelial cells also stimulate EDHFs, which activate calcium-dependent potassium channels to increase intracellular potassium causing hyperpolarization of the endothelial cell membrane. The hyperpolarization is then transmitted to the smooth muscle cells by MEGJs and also causes the potassium to enter the elastic lamina space. Both actions cause smooth muscle hyperpolarization and inhibition of calcium channels resulting in smooth muscle cell relaxation<sup>202</sup>. Increased endothelial intracellular calcium concentrations also cause the production of superoxides from NADPH oxidase, cyclooxygenase, and eNOS. The superoxide can be converted to H<sub>2</sub>O<sub>2</sub> by superoxide dismutase (SOD), which diffuses to smooth muscle cells to activate potassium channels and inhibit calcium<sup>202</sup>. In general vasodilation by EDHF occurs in the distal arterial segments rather than the proximal<sup>201</sup>.



**Figure 8.** Endothelial-dependent relaxing factors (EDRFs) and endothelial-dependent hyperpolarizing factors (EDHF). Image from Kvietys PR, Granger DN. Role of reactive oxygen and nitrogen species in the vascular responses to inflammation. *Free Radic Biol Med.* 2012;52(3):556-592.

Two main features of inflammation appear in the vascular endothelium: increased generation of ROS and decreased availability of NO<sup>200,153</sup>. The endothelial cells in cerebral blood vessels modify the vascular tone in response to the change in the partial pressure of carbon dioxide through a mechanism called chemo regulation<sup>203</sup>. During hypercapnia (increase the partial pressure of carbon dioxide), the endothelial cells produce vasodilators that increase cerebral blood flow, and during hypocapnia the endothelial cells produces vasoconstrictors in form of NOS+ inhibitors and decrease

blood flow<sup>204</sup>, this mechanism is affected by certain vascular disease like subarachnoid hemorrhage, ischemic stroke, and brain trauma<sup>205</sup>.

As discussed earlier, endothelial dysfunction can be caused by reduced NO availability, which can result from decreased eNOS gene expression or L-arginine deficiency<sup>206</sup>, NOS inhibition by asymmetric dimethyl arginine<sup>207</sup>, or caused by uncoupling of eNOS or tetrahydrobiopterin deficiency<sup>208</sup>.

During the childbirth, certain physiological changes take place in the pulmonary blood vessels with the start of ventilation, which is represented in decreasing vascular resistance and increasing blood flow in the pulmonary blood vessels of the neonate with the initiation of ventilation and oxygenation. These physiological changes are attributed to an increase in eNOS gene expression and enzymatic activity and also increase NO production as a result of the enzyme activity<sup>209</sup>. In certain pathological conditions like persistent pulmonary hypertension of the neonates, which result from the failure of the normal physiological process to develop, there is a marked decrease in the mRNA of eNOS in the lung tissue<sup>210</sup>.

Nitric oxide not only acts as a vasodilator, but also has anticoagulant and inflammatory effects, where it inhibits the adhesion of leukocytes to the endothelium of blood vessels in the early stage of inflammation<sup>211</sup>. Moreover, any disturbance in the function of NO in blood vessels may cause hypertension and ischemic stroke which can be due to the impaired vascular effects of NO as well as the disturbance in its antithrombotic effect<sup>212,213</sup>. NO is also involved in other mechanisms involving the regulation of platelet function and the reaction with hemoglobin and reactive oxygen

species<sup>214</sup>. NO modulates platelet-endothelium interactions and maintains blood flow<sup>214</sup>. To maintain blood flow, there are three biochemical systems that suppress platelet activity: prostacyclin, the ecto-AT(D)Pase, and NO. Prostacyclin results from endothelial metabolism of arachidonic acid and inhibits platelet aggregation through the cAMP mechanism while the ect-AT(D)Pase acts through ATP-induced platelet aggregation and through inhibiting thromboxane A2 receptors<sup>215,216</sup>. Since NO exerts its effect through cGMP production and ATPase dependent refilling of calcium stores, this results in lower calcium that is available to interact with platelet aggregation mechanisms<sup>217</sup> causing a decrease in platelet calcium during activation and modification of the shape and cytoskeletal arrangement of platelets, leading to platelet aggregations<sup>218</sup>. NO also decreases platelet plug formation through inhibition of active fibrinogen conformation of glycoprotein resulting in reduced cell receptors required for platelets to interact with each other and form a plug<sup>219</sup>.

NO additionally induces apoptosis of megakaryocytes, which are responsible for platelets formation. Therefore NO controls both the production and function of platelets<sup>220</sup>. Macrophages induced by bacterial endotoxin show elevated levels of NOS and NO, which can have antibacterial effects<sup>221</sup>.

NO produced by eNOS is also a neurotransmitter in the nerves that supply certain blood vessels like cerebral and penile vessels<sup>222</sup>.

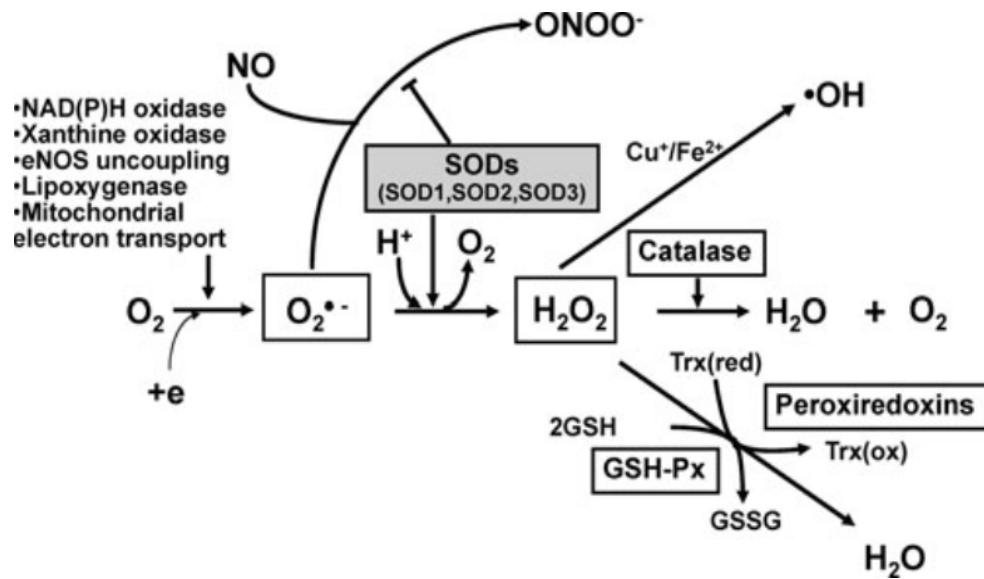
NO can form a complex with the hemoglobin molecule, which is important in hypoxic vasodilation. This process appears during situations of increased oxygen requirement like in certain regions of the brain or to the muscles during exercise. The Hb

molecule becomes S-nitrosylated when it reacts with NO to form SNO-Hb during the process of oxygenation in the lungs<sup>223,224</sup>. This molecule has vasodilator effects and leads to increased blood and oxygen supply to certain tissues during the non-pathological increase of metabolic activity<sup>225</sup>. Disorders of this mechanism are associated with congestive heart failure, pulmonary hypertension, and diabetes<sup>226,227,228</sup>. NO may also react with reactive oxygen species resulting in reduced bioavailability, which is considered one of the two main features of micro vascular dysfunction.

### *Reactive oxygen species*

ROS are the product of aerobic respiration and substrate oxidation during physiological metabolism at the cellular level. Common enzymatic sources of ROS include the mitochondrial electron transport chain, xanthine oxidase, cytochrome p450 monooxygenases, lipoxygenase, NADPH oxidase, and even nitric oxide synthase<sup>229</sup>. The ROS produced include superoxide ( $O_2^-$ ), hydroxyl radical ( $OH^-$ ), hydrogen peroxide ( $H_2O_2$ ), and peroxynitrite ( $ONOO^-$ ). The formation of  $ONOO^-$  from the reaction of NO with  $O_2^-$  is considered the primary source for the production of RNS (reactive nitrogen species), which are considered reactive neurotoxins<sup>230</sup>. These ROS can be used in low concentrations by the body in some normal physiological processes such as against microorganisms as part of the immune system and in intracellular signaling<sup>231,232</sup>. They may also participate in signaling inflammatory chemotaxis immune cells to the site of inflammation<sup>233</sup>, while high concentrations of these ROS or even incomplete removal of them may lead to what is called oxidative stress. Oxidative stress is involved in many pathological conditions like hypertension, diabetes, pulmonary hypertension<sup>234</sup>, and atherosclerosis<sup>235</sup>. Raised levels of ROS affect many biological mechanisms and

pathways like reducing the bioavailability of NO as superoxide ions scavenge NO forming ONOO<sup>-</sup>, leading to endothelial dysfunction. This also affects mitochondrial function, cellular growth through DNA damage through superoxide toxicity or by lipid peroxidation, or through affecting many cellular processes like cell signaling and apoptosis<sup>156</sup>.



**Figure 9.** Generation and metabolism of reactive oxygen species (ROS). Superoxide is produced by NADPH oxidase, xanthine oxidase, nitric oxide synthase (NOS), lipoxygenase, and mitochondrial enzymes, Superoxide is converted by superoxide dismutase (SOD) to H<sub>2</sub>O<sub>2</sub>, which is reduced to water by catalase, glutathione peroxidases (GPx), and peroxiredoxins (Prx). In the presence of reduced transition metal (Fe<sup>2+</sup>, Cu<sup>+</sup>), nitric oxide (NO) can be rapidly inactivated by reaction with O<sub>2</sub><sup>•-</sup> leading to the production of the strong oxidant peroxynitrite (ONOO<sup>-</sup>). Image from Fukai T, Ushio-Fukai M. Superoxide dismutases: Role in redox signaling, vascular function, and diseases. *Antioxid Redox Signal.* 2011;15(6):1583-1606

ROS, especially hydrogen peroxide, can induce endothelial NO activity by inducing eNOS protein synthesis and activity<sup>156</sup>. This increase in activity results in more

NO to react with these ROS<sup>236</sup>. The most important defense mechanism at cellular level against O<sub>2</sub><sup>-</sup> is the superoxide dismutase enzyme (SOD). This enzyme converts O<sub>2</sub><sup>-</sup> into H<sub>2</sub>O<sub>2</sub> with the help of certain metals like copper and manganese as a catalytic metal through oxidation reduction reactions<sup>236</sup>. H<sub>2</sub>O<sub>2</sub> is then transformed to H<sub>2</sub>O by catalase, peroxiredoxins or glutathione peroxidases enzymes<sup>237</sup>. There are three isoforms of SOD that vary in location and genetic formation.

### SOD1

The location of this enzyme is the cytoplasm of the cell<sup>238, 239</sup>. The chromosome that contains the genetic code for this isoform is chromosome 21, which is related to Down syndrome (Trisomy 21). Patients with down syndrome, therefore, have an extra copy of the SOD1 gene that shows increased activity<sup>240</sup>. The metal cofactors for this isoform of the enzyme are Cu<sup>++</sup> and Zn<sup>++</sup> and its half-life is 6-10minutes<sup>241</sup>.

### SOD2

Located in the mitochondria, the main target of SOD2 is O<sub>2</sub><sup>-</sup> that results from the electron transport chain<sup>242</sup>. The metal cofactor of SOD2 is Mn and it has a half-life of 5-6 hours. Deficiency of this isoform leads to disturbances in mitochondrial function in addition to cardiomyopathies and degenerative diseases in mice that lead to death<sup>243</sup>.

### SOD3

This isoform is usually located in the vascular extracellular space and on the surface of smooth muscle cells of the blood vessels, lung, uterus, kidney, and heart tissues<sup>244</sup>. Although it is produced by the vascular smooth muscle, it binds to the

extracellular surface of the endothelial cells<sup>245</sup>. The metal cofactors for this isoform are Cu<sup>++</sup> and Zn<sup>++</sup> and any abnormality in SOD3 can lead to ischemic heart disease in mice<sup>246</sup>.

Endothelial nitric oxide synthase itself can take part in oxidative stress by generating superoxide anions instead of nitric oxide<sup>247,248</sup> through a process called eNOS uncoupling that accompanies many medical conditions like diabetes, hypertension and atherosclerosis<sup>249</sup>. eNOS uncoupling can also result from activation of thromboxane receptors (Tpr) by thromboxane A2, which is an eicosanoid derived from arachidonic acid, is distributed in various tissue in the body and participates in many physiological processes like renal function, immunity, hemostasis, wound healing, vascular tone, as well as other inflammatory conditions<sup>250</sup>. The activation of Tpr by thromboxane A2 was found to increase the production of O<sub>2</sub><sup>-</sup> by vascular smooth muscle cells leading to increased formation of (ONOO<sup>-</sup>) and eNOS uncoupling<sup>251</sup>.

NO is considered an important mediator in many neurodegenerative diseases in the nervous tissue like Parkinson disease<sup>252</sup>, Alzheimer's disease, amyotrophic lateral sclerosis<sup>253</sup>, Huntington chorea<sup>232</sup>, and stroke<sup>254</sup>. NO promotes neurodegeneration through fragmentation of intracellular organelles like mitochondria and the Golgi apparatus which are important in energy production and cellular mitosis resulting in stress and cell death<sup>255</sup>. NO also promotes neurodegeneration by disturbance of the mitochondrial respiratory chain through the inhibition of mitochondrial respiratory complex enzymes.

Two main concepts are related to endothelial dysfunction and the failure of sufficient NO production to cause blood vessel relaxation including diabetic macro



vascular complications like neuropathy, nephropathy, retinopathy, and erectile dysfunction. Diabetic complications also include increased risk of stroke and ischemic heart disease as well as increased severity of these diseases<sup>256,257</sup>.

The possible explanations of these complications include depletion of many intracellular antioxidant enzymes like glutathione peroxidase as a consequence of consumption of NADPH to NADP and reduction of NAD to NADH during the pathological reduction of glucose to sorbitol and oxidation of sorbitol to fructose in diabetic patients. Therefore the cells become prone to stress damage and nitrosative injury<sup>258,259</sup>. Also the increase of intracellular diacylglycerol as a result of increased protein kinase c isoforms reduce endothelial NO synthesis<sup>260</sup>. Antioxidants like superoxide dismutase improve endothelium-dependent vasodilation in blood vessels from individuals with diabetes<sup>261,262</sup>. The increase of intracellular glucose with diabetes increases the release of electrons from the tricarboxylic acid cycle. These liberated electrons increase the proton gradient of the mitochondria increasing the release of superoxide ions<sup>263</sup>.

#### *NO and Diabetes*

Normally, insulin stimulates the uptake of glucose by skeletal muscle and adipose tissues through tyrosine phosphorylation of insulin receptors on the cell surface. This leads to the phosphorylation of insulin receptor substrate-1 (IRS-1) and Shc<sup>264</sup> resulting in the activation of two intracellular pathways:PI3 kinase (PI3K)-AKt, and Ras/Maf/MAP Kinase pathways<sup>265</sup>. In the vasculature, activation of the PI3K pathway, leads to phosphorylation and activation of eNOS while activation of the Ras pathway

causes mitogenic effects as well as increased expression of the endothelial-derived vasoconstrictor endothelin-1<sup>265</sup>.

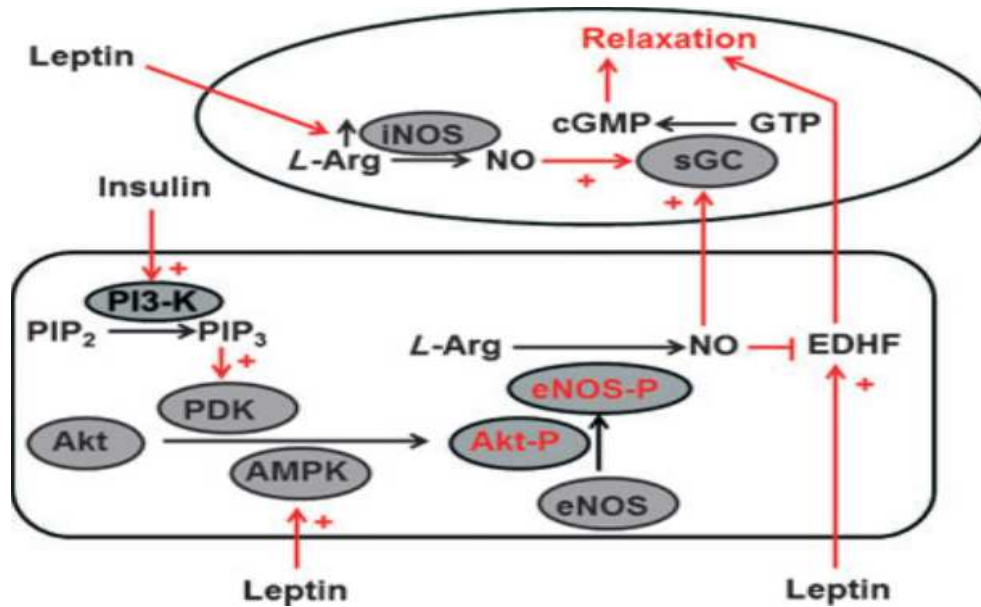
In insulin resistance that accompanies certain clinical conditions like obesity, diabetes, and metabolic syndrome<sup>265</sup>, the balance between the two pathways is disturbed. Activation of the (PI3K)-AKt pathway is impaired leading to decreased NOS activity and NO bioavailability, therefore decreasing insulin dependent vasodilation<sup>266</sup>. In contrast, the function of Ras pathway is usually preserved during insulin resistance resulting in endothelial dysfunction as endothelin-1 concentrations increase<sup>265</sup>. Interestingly, as a result of endothelial dysfunction vasoconstriction develops, disturbing glucose delivery to peripheral tissues by interfering with increased blood flow that would normally result from insulin-induced vasodilation<sup>267</sup>.

In eNOS knock-out mouse models, (low availability of NO in blood vessels wall), blood pressure is 30% higher than in the wild type mice<sup>268</sup>. The absence of eNOS also augments the inotropic response of cardiac muscle and increased cardiac contractility. In contrast, nNOS gene deletion is associated with arrhythmia and left ventricular remodeling after infarction<sup>269</sup>. Moreover, eNOS knock-out mice have enlarged infarction areas in the brain and fail to preserve blood flow to the affected region with a prominent neurological deficit. In contrast nNOS knockout mice have smaller infarction areas suggesting that increased NO production in cerebral ischemia worsens the condition although the presence of NO is proposed to serve important physiological function during the normal conditions<sup>270</sup>.

## *Leptin and NO*

Intravenous leptin administration is associated with an increase in plasma NO concentrations in a time- and dose-dependent manner in rat aortic vessels<sup>271</sup>. On the other hand, central administration of leptin stimulates the sympathetic nervous system which increases the sympathetic outflow to blood vessels and the kidneys in rats<sup>272</sup>. Whereas this should increase blood pressure through vasoconstriction, under normal and healthy conditions, leptin creates a balance between the pressor effect caused by the sympathetic nervous system and the depressor response caused by NO<sup>272</sup>.

Among the evidence that supports the idea that leptin mediates vasodilation through the NO pathway, Limbo et al<sup>273</sup> demonstrated that the administration of leptin to a phenylephrine-constricted rat aortic ring resulted in vasorelaxation. This effect of leptin was abolished by either endothelial denudation of the aortic ring, administration of the nitric oxide synthase (NOS) inhibitor L-NAME (nitro L-arginine methyl ester), or by inhibiting soluble guanylate cyclase<sup>273</sup>.



**Figure 10.** Effects of leptin in the vascular wall of aortic vessels in human. In endothelial cells, leptin stimulates AMP-activated protein kinase (AMPK), which phosphorylates and activates protein kinase Akt. Phosphorylated Akt, in turn, phosphorylates endothelial nitric oxide synthase (eNOS), which generates nitric oxide (NO). NO activates soluble guanylate cyclase (sGC), which synthesizes cGMP in smooth muscle cells to induce vasorelaxation. Endothelial NOS may also be activated by insulin, which stimulates the phosphoinositide 3-kinase (PI3-K) pathway. Leptin stimulates iNOS to produce NO causing vasorelaxation beside its role in EDHF which also results in vasorelaxation in leptin sensitive blood vessels. This effect is diminished in obese people and may contribute to hypertension. Image from Beltowski J. Leptin and the regulation of endothelial function in physiological and pathological conditions. *Clin Exp Pharmacol Physiol.* 2012;39(2):168-178.

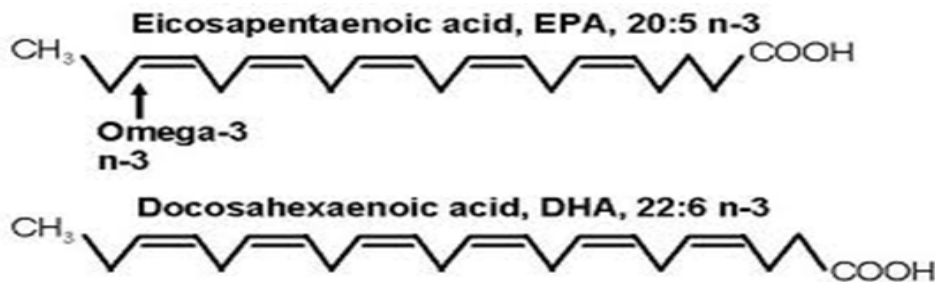
Insulin stimulates insulin receptors on vascular endothelial cells resulting in phosphorylation of insulin receptor substrates (IRS1, IRS2) resulting in stimulation of the phosphoinositide 3-kinase (PI3K) pathway causing Akt to stimulate NO production from eNOS<sup>274</sup>. Leptin binding to its receptor activates the same signaling pathway in the endothelial cells of rats without stimulating insulin receptors<sup>275</sup>. Moreover, leptin

increases Akt phosphorylation and eNOS stimulation in aortic rings obtained from rats<sup>276</sup> through the Akt pathway in the endothelial cells without activation of PI3K.

This information is important in understanding the development of hypertension in obese patients with hyperleptinemia as the anorectic effect of leptin in the hypothalamus is impaired while the sympathetic nervous system effect of leptin is over-stimulated leading to the development of hypertension. Moreover, chronic hyperleptinemia, which accompanies leptin resistance is associated with the generation of ROS and oxidative stress also contributing to vascular pathologies<sup>277</sup>.

### *Omega 3*

Omega 3 is a polyunsaturated fatty acid with a double bond c=c located at the carbon atom number 3 in the chain from the methyl end. This position of the double bond makes it unaffected by any metabolism and transformation processes that take place inside the body. This bond is also more energy stable, and any other compound synthesized from these fatty acids will be obtained from changes taking place at carboxylic acid end or from double bonds located in the middle of the chain<sup>278</sup>.



**Figure 11.** The structure of omega 3 fatty acids. Image from [www.louisville.earthsave.org](http://www.louisville.earthsave.org)

Omega 3 fatty acids include three types of fats that range from alpha linoleic acid which comes from plant source like flaxseed oil, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) which are mainly obtained from fish oil<sup>278</sup>. These fatty acids are considered essential fatty acids because the human body cannot synthesize them<sup>279</sup>. Therefore, they can only be obtained from dietary sources. In addition, humans have a limited ability to convert alpha linolenic acid (ALA) which contains 18 carbon atoms in its structure and 3 double bonds by the process of elongation into EPA with 20 carbon atoms and 5 double bonds and then into DHA that contains 22 carbon atoms and 6 double bonds. In fact, studies showed that the rate of conversion of ALA to EPA varies between 0.2% and 21% and that of ALA to DHA varies between 0% and 9%<sup>280</sup>, although this ability is more efficient in women compared to men<sup>281</sup>. In these fatty acids the double bonds all lie in cis configuration, which means that the two hydrogen atoms lie on the same side of the bond<sup>282</sup>.

### *Dietary source*

The adequate intake (AI) level of omega 3 is 1.6 grams /day for men and 1.1 grams / day for women and the Acceptable Macronutrient Distribution Range (AMDR) is 0.6%-1.2% of total energy<sup>278</sup>. However it is reported that median consumption of EPA + DHA by Americans is between 0 and 1 g/d of ALA<sup>283</sup>. This can be explained by a general reduction in fish intake, increased consumption of domestically- raised fish in comparison to ocean fish, and less omega 3 fatty acids available in commercial foods<sup>284</sup>.

It is estimated that about \$33.9 billion is spent annually on alternative medicine in the United States of America alone. Of this, \$14.8 billion was spent on nutritional

supplements. Omega -3 supplements (including fish oils) were reported as the most common non vitamin / non mineral product taken by adults<sup>285</sup>. It is recommended by the FDA that an adult should consume about 3 grams, on average of DHA and EPA daily. The American Heart Association recommends that patients with ischemic heart disease should consume fish oil two times a week which may increase to one gram of fish oil daily following a myocardial infarction and up to 2-4 grams daily to lower triglyceride levels<sup>278</sup>.

In addition to the effect of omega 3 in the cardiovascular system, omega 3 fatty acids show various health benefits by improving blood pressure control, alleviating symptoms of rheumatoid arthritis and depression, as well as attenuating the progression of Alzheimer's disease<sup>286,287,288,289</sup>. In general, omega 3 plasma levels are negatively associated with BMI, WC (waist circumference) as well as HC (hip circumference) in healthy and obese subjects<sup>290</sup>. Bhagavathi et al<sup>291</sup> showed that the combination of omega 3 fatty acids with synthetic organoselenium ( 1,4-phenylene bis(methylene) selenocyanate(p-XSC) ) can help suppress colon cancer growth and induce apoptosis of tumor cells and through inhibition of COX-2 and iNOS activity in colon tumors.

### *Fish and fish oil*

Fish are considered an important nutrient source of omega 3. Fish can be divided into lean fish that store fat in the form of triacylglycerol in the liver, and oily fish that store fat in form of triacylglycerol in the flesh<sup>292</sup>. Cold water oily fish like sardines , salmon , herring , and anchovies are important sources of omega 3 fatty acids, however , these fish do not synthesize omega 3 in their body, but instead acquire it from their food

(algae or plankton)<sup>293</sup>. These omega 3 fatty acids concentrated in fish are, accumulated with high quantities of antioxidants like iodide and selenium which protect omega 3 from peroxidation. However, there are some concerns about fish oil intake from some species of fish like swordfish, sharks, and albacore tuna due to the possibility of toxic contamination by mercury, and dioxin<sup>294</sup>. Other sources of omega 3 include flaxseeds, kiwifruit, black raspberry, nuts, soybeans, linseeds, mustard, and canola<sup>295</sup>.

Omega 3 fatty acids are metabolized by cytochrome P450 (CYP450) enzymes to their metabolites (epoxyeicosatetraenoic acid (17, 18-EEQ) and hydroxyeicosapentaenoic acid (20-HEPE) for EPA) and (epoxydocosapentaenoic acid (19, 20-EDP) and hydroxydocosahexaenoic acid (22-HDoHE) for DHA)<sup>296</sup>. Aspirin-acetylated cyclooxygenase-2 (COX-2) and 5-lipoxygenase enzymes also convert EPA to 18S- and 18R-resolvins E1 and E2<sup>297</sup> and aspirin-acetylated COX-2 converts DHA to 17R-resolvins D1–D4 and 17R- protectin D1, and by 5- and 15-lipoxygenase 17S-group of metabolites<sup>298,299</sup>. These metabolites vary in their biological activity in comparison to the parent omega 3 fatty acids. For example, DHA shows weaker potency in relaxation of constricted arterioles through the activation of large-conductance calcium-activated potassium (BKCa) channels in comparison to the metabolites of cytochrome P450 enzymes<sup>300</sup>, while EPA shows stronger effects in comparison to its metabolites from the same enzyme groups<sup>301</sup>.

Polyunsaturated fatty acids, including omega 3 fatty acids, are affected by the influence of ROS that interact with PUFA in cell membrane or lipoproteins through a process of uncontrolled peroxidation. Malondialdehyde, one of the end products of this peroxidation, has adverse effects in the human body<sup>302</sup>.



Omega 3 intake is associated with increase concentrations of EPA and DHA in the phospholipids of the cell membrane that include structures such as phosphatidylcholine and phosphatidylserine<sup>303</sup>. This effect leads to decreased liberation of arachidonic acid from phospholipid that can be used by COX and lipoxygenase enzymes to produce the 2-series prostaglandins, and 4-series leukotrienes<sup>304</sup>. However, omega 3 in fish oil intake by normal subjects showed incorporation of omega 3 in platelets phospholipid decreasing platelets count and function<sup>305</sup>. Omega 3 fatty acids also take part in the formation of diacylglycerol, which is a lipid molecule that takes part in cell signaling and activation of protein kinase class c (PKC)<sup>306</sup>.

In addition to their functions as a part of cell membrane phospholipids, omega 3 act as ligands to various receptors like G-protein coupled receptors, GPR120, that are present in gastrointestinal epithelial cells and macrophages. Activation of this group of receptors will leads to the secretion of glucagon-like peptide-1 from epithelial cells<sup>307</sup> and anti-inflammatory effects in macrophages and monocytes through the activation of (GPR120)<sup>308</sup>. and by activation of many nuclear receptors like retinoid-activated nuclear receptor (RXR $\beta$ )<sup>285</sup> and peroxisome proliferator-activated receptor (PPAR) which is responsible for gene activation and synthesis of adiponectin<sup>309</sup>, while inhibiting the activity of the transcription factor, nuclear factor kappa B (NF $\kappa$ B)<sup>310</sup>.

### *Omega 3 and Cardiovascular System*

Omega 3 intake negatively affects mechanisms involved in the process of cardiovascular disease including atherosclerosis and factors that can eventually causes myocardial infarction and stroke<sup>311,312</sup>. These mechanisms include reduction in serum

triacylglycerol levels up to 30% following intake of 2 grams of fish oil daily<sup>313</sup> and reduction in chemo-attractant, growth factor, and adhesion molecules<sup>314,315</sup>, which are important in enhancing leukocytes and smooth muscle migration in the inner layer of the blood vessels wall starting the process of vascular inflammation<sup>316</sup>.

Omega 3 is effective at decreasing blood pressure in both normo and hypertensive subjects<sup>317</sup> and affects vascular endothelium relaxation through promotion of NO production which will be discussed later. In addition to the effect on atherosclerosis, omega 3 show preventive effects against factors that lead to acute cardiovascular events like myocardial infarction and stroke. This effect is mediated by the anti-thrombotic effects of omega 3 to decrease the production of TXA2 (a strong platelet aggregator) and PGI3 by COX-2 from the arachidonic acid, that is released from the phospholipids in the cell membrane by the effect of phospholipase A2<sup>305,318</sup>. This effect is modulated through the incorporation of omega 3 into the cell membrane phospholipids by phosphatidylethanolamine instead of arachidonic acid and when released by phospholipase A2, it leads to the formation of PGI3 and TXA3 which reduce platelet aggregation<sup>318</sup>.

Omega 3 show anti- arrhythmic properties through decreasing the electrical excitability and ion modulation in cardiomyocytes as a result of increased omega 3 concentrations inside the phospholipid bilayer of the cell membranes<sup>319</sup>.

Omega 3 shows the ability to decrease infiltration of inflammatory cells into atherosclerotic plaques and to decrease the activity of cells already present inside the plaques<sup>320</sup>. In addition, omega 3 affect the expression of some adhesion molecules like

ICAM-1 (intercellular cell-adhesion molecule-1) and VCAM-1 (vascular cell adhesion molecule-1) on the surface of endothelial cells<sup>321</sup>. These molecules are glycoproteins synthesized in response to the raise in the inflammatory cytokines. They are expressed from activated endothelial cells and are released by proteolytic shedding from the surface of the cells. Adhesion molecules stimulate the adhesion of monocytes to the endothelium cells and the uptake of oxidized low density lipoproteins to form foam cells in the sub-endothelial space thereby initiating atherosclerosis through the inhibition of NF- $\kappa$ B (nuclear factor kappa B transcription factor ) activation, which regulates the synthesis of inflammatory molecules and cytokines<sup>321</sup>. In addition, omega 3 decreases the production of chemo attractants like monocyte chemo attractants protein 1 (MCP-1) and platelet derived factor<sup>322</sup>, which lead to stabilization of plaques and prevent their rupture.

The balance in intake between omega 3 and omega 6 fatty acids is considered an important determinant in cardiovascular health. A low ration of omega 3 to omega 6 is considered a potential risk factor for the cardiovascular system and a provoker for inflammation status in the body<sup>323</sup>. Therefore, it is important to have 1:4 of omega 3 and omega 6. However, it is concerning that this ratio is often up to 1:15 in developed countries<sup>323</sup>.

### *Omega 3 and Diabetes*

The daily consumption of omega 3 is associated with low prevalence of type 2 diabetes mellitus<sup>324</sup>. This can be explained by the fact that omega 3 is considered an important component of the phospholipid bilayer in the cell membrane structure. Therefore, omega 3 can affect the transduction of insulin signals through the cell

membrane<sup>324</sup>. In addition, omega 3 fatty acids are involved in controlling many genes related to glucose metabolism<sup>325</sup>. Omega 3 may also decrease insulin resistance through decreasing serum triglyceride and small dense lipoproteins<sup>326</sup>. Moreover, it was found that substituting short chain fatty acids with omega 3 fatty acids has a positive effect on insulin sensitivity and impaired glucose tests<sup>327</sup>. Ramel et al.<sup>327</sup> also showed that consumption of 1.3 grams daily of fish oil for 8 weeks decreased fasting blood glucose and insulin resistance in overweight and obese participants.

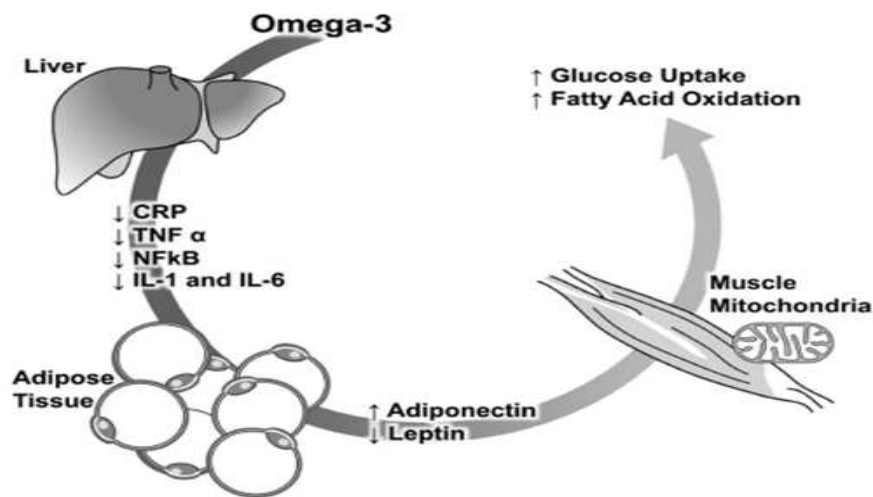
Atherosclerosis and coronary heart disease are among the long term complications of diabetes mellitus, and it was concluded that diabetic patients supplemented with EPA-DHA, have lower fatal CHD rate and the incidence of CHD is decreased with increased omega 3 intake<sup>328, 329</sup>.

### *Leptin and Omega 3*

In general, omega 3 intake leads to healthy weight and prevents abdominal adiposity<sup>290</sup> and omega 3 intake is associated with low body mass index and low waist and hip circumferences<sup>290</sup>. Supplementation of healthy subjects with omega 3 also results in a significant decrease in fat mass without altering body weight<sup>330</sup>. In contrast, in obese or overweight diabetic women, omega 3 intake decreases both total fat mass (mainly truncal fat mass) and subcutaneous adipocyte diameter<sup>331</sup>.

Serum leptin is negatively correlated with the level of dietary omega 3 intake<sup>332,333</sup> and leptin expression and concentration are decreased following intervention with fish oil and/or long-chain (n-3) PUFA<sup>334</sup>. Moreover, Perez et al<sup>334</sup> demonstrated that omega 3 supplementation to rats gaining weight on continuous high energy diets can

actually increase leptin mRNA expression. Leptin secretion is also negatively correlated with glucose metabolism, a relationship that is not influenced by insulin<sup>334</sup>. Also, Pieke et al<sup>335</sup> showed that serum leptin levels decreased in hypertriglyceridemic patients after dietary SFA had been replaced by marine n-3-PUFA and MUFA. This effect can be attributed to omega-3 PUFA accumulation in adipose tissue<sup>336</sup>, which may incorporate into the membrane phospholipid fraction and TG lipid droplets of adipocytes<sup>337</sup>. This causes an alteration in fat tissue endocrine activity, as well as prevents adipose tissue inflammation<sup>338</sup> and adipose tissue matrix remodeling<sup>339</sup>. Moreover, leptin receptor (LEPR) polymorphisms associated with increased insulin resistance (a key feature of metabolic syndrome) are more evident in individuals with low plasma (n-3) or high plasma (n-6) PUFA<sup>340</sup>.



**Figure 12.** Effect of omega 3 on leptin and adipose cells. Image from Pieke B, von Eckardstein A, Gulbahce E, et al. Treatment of hypertriglyceridemia by two diets rich either in unsaturated fatty acids or in carbohydrates: Effects on lipoprotein subclasses, lipolytic enzymes, lipid transfer proteins, insulin and leptin. *Int J Obes Relat Metab Disord.* 2000;24(10):1286-1296.

### *Omega 3 and NO*

NO synthesis in the endothelium of blood vessels is affected by the quantity and composition of fatty acid intake in the diet. Omega 3 have been shown to increase endothelial NO synthase activity in aortic rings through the induction of calcium mobilization and calmodulin system which in turn increases the NO production and its vasorelaxant activity in endothelial cells<sup>341</sup>. These effects are in addition to the translocation of eNOS from specialized plasmalemmal membrane micro domains called caveolin to the cytoplasm following mechanical stimuli<sup>341</sup>. Moreover, Stebbins et al<sup>342</sup> showed that long term intake of DHA will increase the expression of certain proteins like Akt kinase and heat shock proteins 90 that cause an increase in eNOS activity in response to shear stress or endothelial growth factor. This enhanced enzyme activity increases NO production up to 3- folds<sup>343</sup>. However, diets high in long chain fatty acids increase oxidative stress and decrease NO production in the endothelial cells even in the presence of healthy levels of antioxidants<sup>344</sup>.

## CHAPTER 3

### METHODS

This study is a secondary analysis of samples collected during a previous study conducted by Megan Gutierrez and Bianca Teran as a part of their master's thesis while attending Arizona State University in 2013 (Fish oil supplements and symptoms of the common cold in healthy young women , Omega-3 supplementation and body weight in healthy young women). An informed written consent was obtained from all participants, and the Institutional Review Board at Arizona State University approved the study.

The subjects in the original study were healthy, normal weight women between the ages of 18-38 years from at Arizona State University. Subjects were recruited through emails using the Arizona State University listserv. Eligibility of those that responded to the email was determined based on diet, physical activity, smoking habits, brief medical history, and self-reported BMI. Exclusion criteria included regular smoking (use of >10 cigarettes per day), BMI >30 or <18.5, regular intake of omega-3 or fish oil supplements, vegetarian dietary patterns (exclusion of all fish, meat, and poultry from the diet), regular consumption of one or more 3.5oz servings of fish per week, and/or use of prescription medications that may have a potential effect on body weight or affect the inflammatory state (such as corticosteroids or non-steroidal anti-inflammatory drugs). Women were also excluded if they were pregnant or lactating, as well as competing and/or training athletes, or on birth control for less than 3 months. Furthermore, subjects with any

unresolved health issues were not included in the study. Finally, anyone who has had the seasonal flu shot was excluded because of an accompanying study.

Subjects who were deemed eligible were contacted for the first screening interview. During this visit participants completed a written consent form, completed a brief medical history questionnaire, and a validated omega-3 fatty acid food frequency questionnaire (FFQ). Anthropometric measurements, including weight, height, and percent body fat, were also measured at this visit. Height and weight were measured using a stadiometer and calibrated scale. BMI and percent body fat were measured using a bioelectrical impedance scale (Tanita).

Qualifying subjects entered the 8-week trial and were randomly assigned to either the experimental (fish oil, FISH) or control (placebo, CON) group. Random assignments to groups were performed by a coin toss.

During the second visit, estimated dietary and calorie intake were obtained by a 24 hour recall and participants received supplements based on double-blind procedures to prevent bias. Participants received either placebo (Puritan's Pride brand, coconut oil soft gels, 1000mg each) or fish oil capsules (Energy First brand, USP-certified fish oil soft gels, 400mg EPA + 200mg DHA/capsule), which were similarly-sized gel capsules. Participants were instructed to ingest one capsule in the morning, preferably with food, and check off each day that a capsule was taken on a provided calendar. Participants were also given booklets containing validated Godin Leisure-Time Exercise questionnaires to complete weekly, and were instructed to document the use of alcohol, cigarettes, medications, and supplements each day of the trial period. All questionnaires were



explained to the subjects, and a trial-run was completed. Height, weight, BMI, and percent body fat measurements were also taken during this “baseline” visit (using a stadiometer and a bioelectrical impedance scale, Tanita) and a fasting blood sample was collected.

Measurements taken weeks 4 and 8 included body weight, percent body fat, and BMI measurements. Unused capsules from the previous weeks were returned during these visits using a double-blind procedure, and remaining study materials (booklets containing physical activity log and capsules for weeks 5-8) were distributed during the week 4 visit. A final 24-hour dietary recall and fasting blood sample were also collected during the final visit (week 8).

Compliance to capsule administration was monitored through capsule counts at weeks 4 and 8. Biweekly emails from a blind master list were also sent to all subjects to check for compliance to the study protocol.

A total of 35 subjects were enrolled in the study, allowing for 18 subjects to be in the fish oil group (FISH) and 17 subjects in the coconut oil placebo group (CON). However, a total of 9 subjects dropped out throughout the study, leaving 13 subjects in each group (for a total of 26 subjects) that completed the study.

### **Laboratory analyses**

Plasma leptin concentrations were measured using a commercially available kit (eBioscience, Cat. BMS2039INST) according to the manufacturer's directions (see Appendix A). However, since there was a 20% possibility for measuring error with using this kit, we used leptin measurements from the original study in the statistical analysis.

Plasma nitrate and nitrite concentrations were measured on samples collected at baseline and at week 8 of study using a commercially available kit (Cayman Chemical Company, Cat. 780001) following the manufacturer's methods (Appendix B).

Plasma superoxide dismutase activity was measured at baseline and at the end of the study (week 8) according to the manufacturer's protocol (Appendix C) using a commercially available kit (Cayman Chemical Company, Cat. 706002).

### **Statistical Analyses**

The data were analyzed using SPSS Statistical Software. Mann-Whitney U test was used to compare leptin, NO, and super oxide dismutase levels at the beginning, and end of the 8 week study between the omega 3 and control groups. Spearman correlations were used to determine if there was an association between serum leptin and NO concentrations. Mean, median, and standard deviations were measured for all variables. (p-values of  $\leq 0.05$ ) were considered statistically significant.

## CHAPTER 4

### RESULTS

Twenty six subjects (13 in fish oil group and 13 in control group) completed the study. All baseline data are presented in Table 1. Mean participant age at baseline was  $24 \pm 5$  years for the fish oil group, while it was  $23 \pm 6$  years for the control group. Mean BMI at baseline was  $23.2 \pm 2.9$  for fish oil group and  $24.2 \pm 3.3$  for control. Baseline mass weight for the fish oil group was  $140.7 \pm 22.0$  and  $147.6 \pm 23$  pounds for the control group. Percent body fat at baseline for the fish oil group was  $28.4 \pm 7.0$  and  $30.1 \pm 6.6$  for control group.

Mean leptin concentrations at baseline were  $13.8 \pm 8$  and  $16.2 \pm 7.5$  ng/ml for the fish oil group and control groups, respectively. Mean concentrations of NO in the fish oil group was  $11.1 \pm 8.2$   $\mu$ M in comparison to  $10.1 \pm 3.0$   $\mu$ M in control group. The mean level for SOD at baseline for fish oil group showed  $3.30 \pm 1.8$  U/ml, while it was at  $3.05 \pm 1.3$  U/ml in the control group.

	FISH OIL	RANGE	CONTROL	RANGE	*P VALUE
Subjects , n	13		13		
AGE,Y	$24 \pm 6$	18-38	$23 \pm 6$	18-38	0.362
BMI	$23.2 \pm 2.9$	19.5-29.9	$24.2 \pm 3.29$	18.8-29.6	0.362
WEIGHT,LB	$141 \pm 22$	108-185	$148 \pm 23$	111-199	0.545
BF%(body fat percent)	$28.4 \pm 7.0$	15.0-38.9	$30.1 \pm 6.6$	19-39.9	0.687
LEPTIN	$13.8 \pm 8.6$	3.35-27.9	$16.2 \pm 7.5$	3.44-29.3	0.479
NO $\mu$ M	$11.1 \pm 8.2$	4.90-37	$10.1 \pm 3.0$	5.50-17.1	0.614
SOD U/ml	$3.30 \pm 1.8$	2.15-9.15	$3.05 \pm 1.2$	2.01-6.90	0.614

\*p-value represents Mann-Whitney U

All data for participants at the end of the study (week 8) are presented in Table 2). At the end of the study mean BMI for fish oil group was  $23.1 \pm 3.1$  in comparison to  $24 \pm 3$  in control group. The mean body mass for fish oil group at the end of the study was  $140 \pm 23$  while it was  $147 \pm 23$  pounds for those in control group. The mean percent body fat was  $29.04 \pm 6.9$  for fish oil group and  $30.8 \pm 6.3$  for control group.

Mean leptin concentrations in the fish oil group at the end of the study was  $15.3 \pm 9.0$  in comparison to  $17.2 \pm 8.24$  ng/ml for the control group at the end of the study, while mean NO levels at the end of 8 weeks for fish oil group and control group were  $10.1 \pm 5.9$   $\mu$ M and  $11.1 \pm 6.0$   $\mu$ M, respectively. Lastly, mean SOD levels for the fish oil group were  $3.53 \pm 1.6$  U/ml in comparison to  $3.07 \pm 1.0$  U/ml for control group at the end of the study (8 weeks).

<b>Table2.</b> Subject characteristics at week 8					
	FISH OIL	RANGE	CONTROL	RANGE	P VALUE*
Subjects , n	13		13		
BMI	$23.1 \pm 3$	19.3-30.4	$24.0 \pm 3.3$	18.4-28.9	0.362
WEIGHT, LB	$140 \pm 2$	108-188	$146 \pm 23$	115-199	0.448
BF	$29.0 \pm 6.9$	15.0-39.9	$30.8 \pm 6$	19.9-40	0.687
LEPTIN	$15.3 \pm 9.08$	3.59-33.0	$17.2 \pm 8.2$	4.10-31.1	0.687
NO $\mu$ M	$10.1 \pm 5.9$	5.29-28.8	$11.1 \pm 6.0$	4.98-21	0.960
SOD U/ml	$3.53 \pm 1.6$	2.73-8.70	$3.07 \pm 0.96$	2.07-5.69	0.287

\*p-value represents Mann-Whitney U

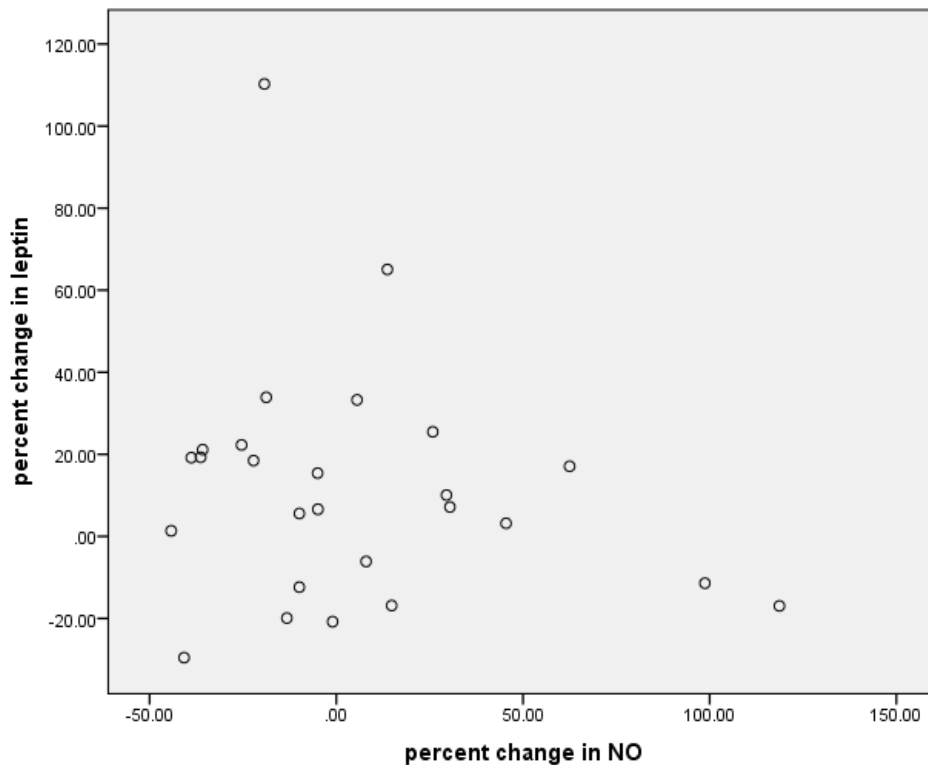
The percent change in data from baseline to week 8 is presented in Table 3. The percent change of leptin showed an increase in both groups at the end of the study. Although not significant, the fish oil group appeared to increase more than the controls ( $15.3 \pm 31.9$  for fish oil group,  $7.83 \pm 27$  for control group; p value was 0.763). The percent change in SOD for the fish oil group was  $11.94 \pm 20.94$  in comparison to control group  $11.8 \pm 53.9$ . However these changes were not significant (p=0.362). NO percent change

was  $-1.97 \pm 22$  in fish oil group while it was  $11.8 \pm 53.9$  in the control group. This change was not significant ( $p=0.960$ ).

<b>Table 3.</b> Percent change in plasma leptin, NO, SOD after 8 weeks of fish oil or placebo supplementation			
	Fish Oil	Control	P-value*
Leptin,	$15.30 \pm 31.88$	$7.83 \pm 26.8$	0.763
SOD	$11.94 \pm 20.94$	$3.79 \pm 19.14$	0.362
NO	$-1.97 \pm 22.57$	$11.78 \pm 53.86$	0.960

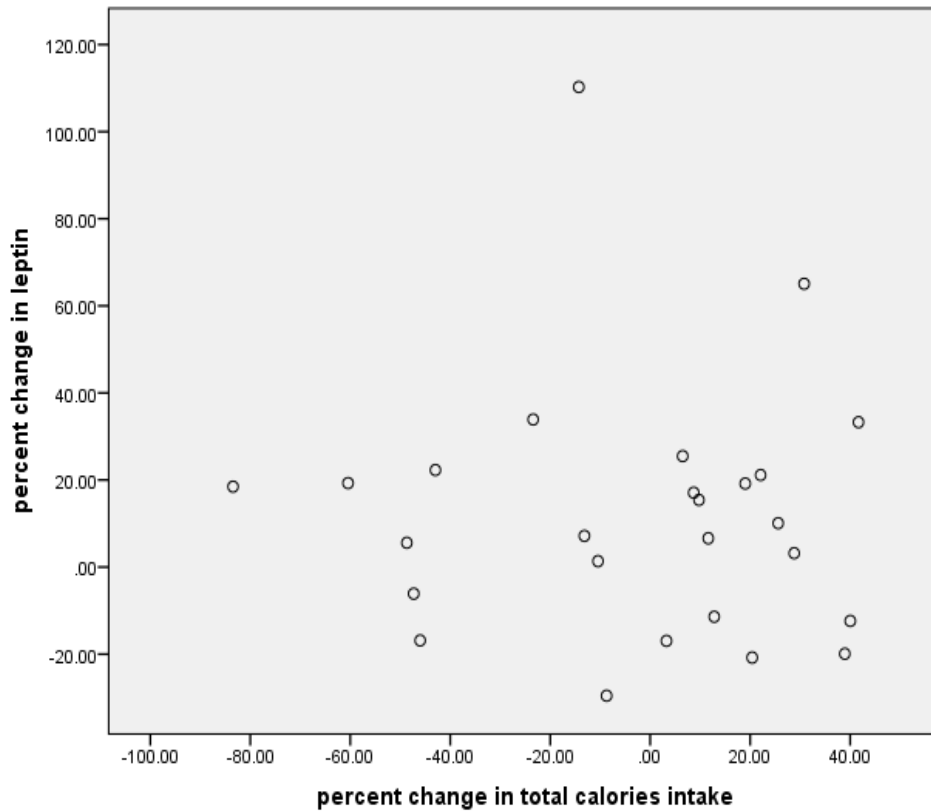
\*p-value represents Mann-Whitney U

The Pearson correlation co-efficient between the percent change of both leptin and NO was  $r^2 = -0.251$ .



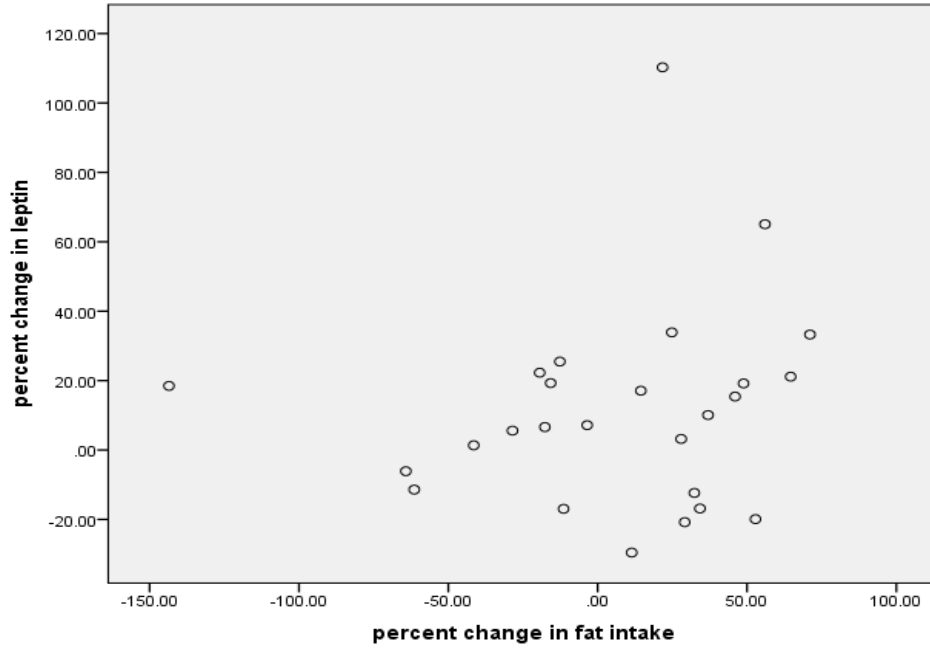
*Figure 13.* Correlation of change in leptin with change in NO. The correlation co-efficient was  $r^2 = -0.251$ .

The Pearson correlation co-efficient between percent change in leptin and total calories intake was  $r^2 = -0.039$

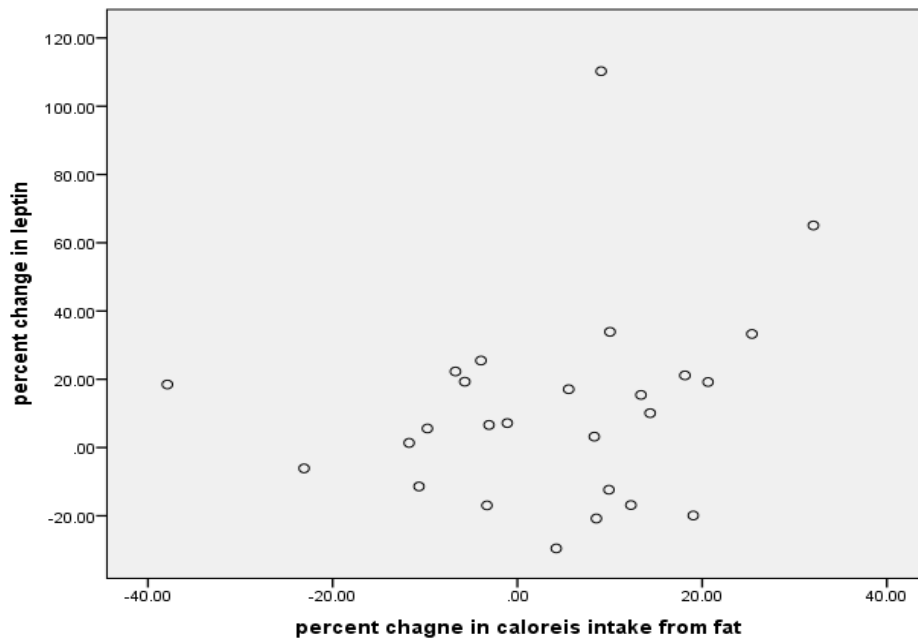


*Figure 14. Correlation of percent change in leptin and the percent change in total caloric intake. The correlation co-efficient was  $r^2 = -0.039$ .*

The Pearson correlation co-efficient between percent change of leptin and percent change in total fat intake was  $r^2 = 0.156$  and the Pearson correlation co-efficient between percent change of leptin and percent change in total calories from fat intake was  $r^2 = 0.231$



**Figure 15.** Correlation of percent change in leptin with the percent change in fat intake. The correlation co-efficient is  $r^2=0.156$ .



**Figure 16.** Correlation of percent change in leptin with percent change in caloric intake from fat. The correlation co-efficient is  $r^2=0.231$ .

## CHAPTER 5

### DISCUSSION

Subjects showed no significant difference in their descriptive characteristics at baseline and at the end of the study (Tables 1, 2). Both the omega 3 group and control group showed no marked difference in their weight or body fat percent and although not significant, BMI tended to increase in both groups. Leptin hormone showed a slight increase at the end of the study in comparison to its level in both groups at baseline, a similar pattern was evident in NO in both groups, while SOD showed no marked difference between week 0 and week 8 in either group.

The percent change in leptin between week 0 and week 8 increased in both groups, although the standard deviation was high. Leptin tended to increase more in the omega 3 group in compare to control (Table 3), although the change in leptin was not significant. These findings are in contrast to prior studies that have shown decrease in leptin following regular intake of 300-600 mg of omega 3<sup>345</sup> as leptin and omega 3 are negatively correlated<sup>332</sup>.

SOD percent change in both groups was increased with a marked high standard deviation in both groups but the change, although not significant, was greater in the omega 3 group (Table 3). These findings are similar to prior studies that have shown an increase in SOD activity following omega 3 intake<sup>15</sup> and a significant increase in SOD levels<sup>346</sup>.

Percent change in NO in the omega 3 group declined by the end of the study while it increased in the control group; however the standard deviation was large in both groups (Table 3). Other studies have shown that omega 3 supplementation increases the



production of NO and NOS activity in blood vessels<sup>341</sup>. However high supplementation of long chain fatty acids (11.5 % of total energy intake for 4 weeks) can lead to increased oxidative stress and eventually decreased availability of NO<sup>344</sup>. In contrast to the hypothesis that leptin and NO would be positively correlated<sup>271</sup>, a weak negative association was found in the current study ( $r^2 = -0.251$ ).

In addition the Pearson correlation co-efficient between percent change in leptin level with the percent change of fat intake was weakly positive ( $r^2 = 0.156$ ). The percent change of total caloric intake was also weakly negative ( $r^2 = -0.039$ ) and total caloric intake from fat was weakly positive ( $r^2 = 0.231$ ). Our findings were similar to a prior study that showed a positive association between leptin and total caloric intake from fat<sup>347</sup>.

## CHAPTER 6

### CONCLUSION

In conclusion, the results of the study accepted the first null hypothesis as leptin hormone concentrations did not change significantly following 8 weeks of omega 3 supplementation (600 mg/daily) in healthy adult female subjects. Although the omega 3 and control groups appeared to show an increase in leptin, this increase did not reach significant levels. Leptin levels at the baseline of this study were within normal levels for healthy non obese subjects and leptin level tend to increase with increasing body fat mass. Therefore, low dose of omega 3 on healthy subjects in this study did not show significant results.

In this study NO levels did not significantly change following the same intervention protocol. Therefore the second null hypothesis can be accepted. The third null hypothesis was also accepted in this study as SOD levels did not change significantly following the omega 3 treatments. Similarly, the fourth null hypothesis was accepted since the study did not find a significant association between leptin and NO levels.

The negative findings in this study may be due to the fact that the subjects who participated in the study were healthy and not obese nor did they report any health conditions known to raise oxidative stress or inflammation. Since leptin is an adipose derived hormone, normal weight individuals would be expected to have lower levels than obese individuals. In contrast to observations in rabbits<sup>16</sup>, omega 3 supplementation did not appear to upregulate leptin expression resulting in greater release in the current protocol.

Understanding the mechanism of weight control in the body and the elements that participate in this process like leptin hormone is very important in the management and prevention of a common health concern like obesity. The concept of endothelial dysfunction and its relation to cardiovascular disease can be explored further and clarified through better understanding of the function of NO and SOD enzyme in vascular function and oxidative stress.

Omega 3 is an important poly unsaturated fatty acid that has protective effects in the cardiovascular system and can decrease fat mass in the body. A larger sample size that includes healthy males or overweight male and female subjects may have resulted in different outcomes since obesity and overweight are associated with increase the level of inflammation and oxidative stress and associated with marked increase in leptin levels. Moreover, a higher dose of omega 3 fatty acids may have elicited greater effects than those observed in the prior study. Future studies in this area should therefore include male subjects as well as overweight subjects administered larger doses of fish oil that are equivalent to three or more servings per week as opposed to the two servings implemented in the current study. The importance of gender cannot be underestimated since estrogen has protective effects in the vasculature of females that may have masked any further protective effects of the fish oil.

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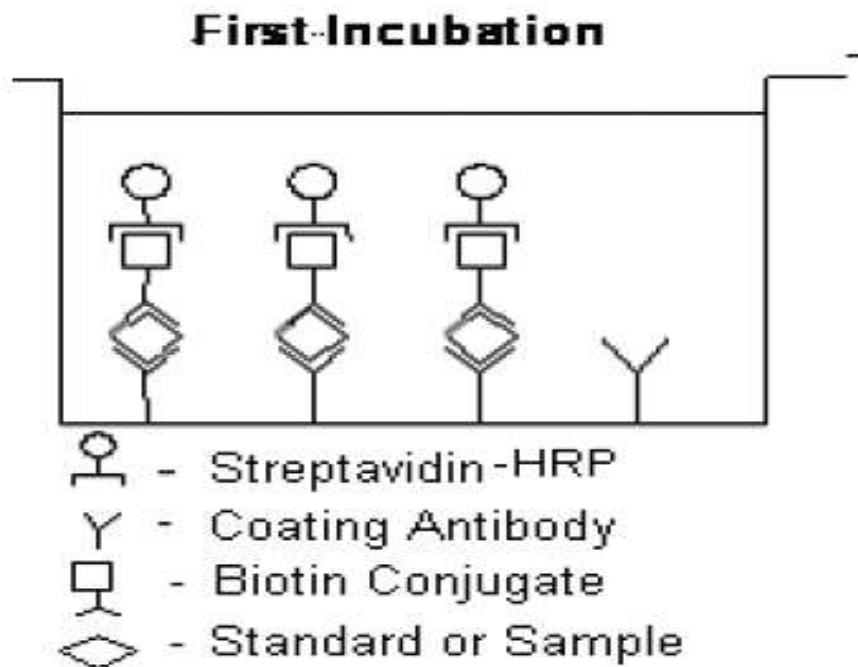
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## APPENDIX A

### LEPTIN ASSAY PROTOCOL

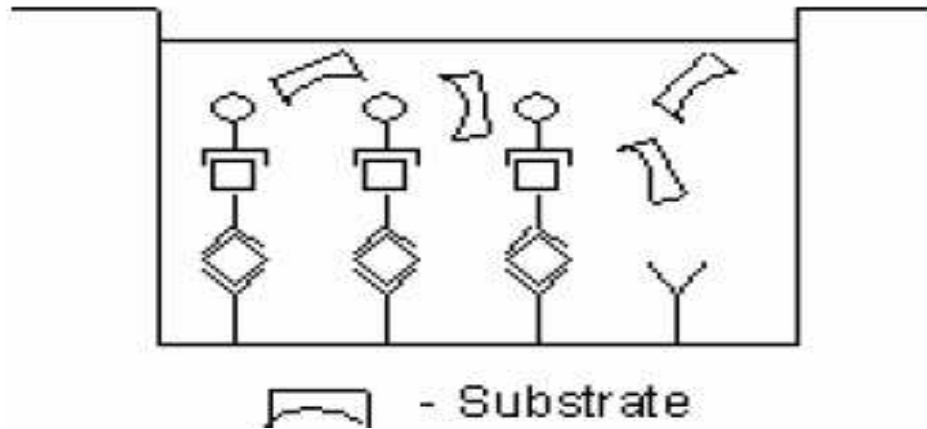
## Principles of the Test

An anti-human Leptin coating antibody is adsorbed onto microwells. Human Leptin present in the sample or standard binds to antibodies adsorbed to the microwells; a biotin-conjugated anti-human Leptin antibody binds to human Leptin captured by the first antibody. Streptavidin-HRP binds to the biotin conjugated anti-human Leptin.

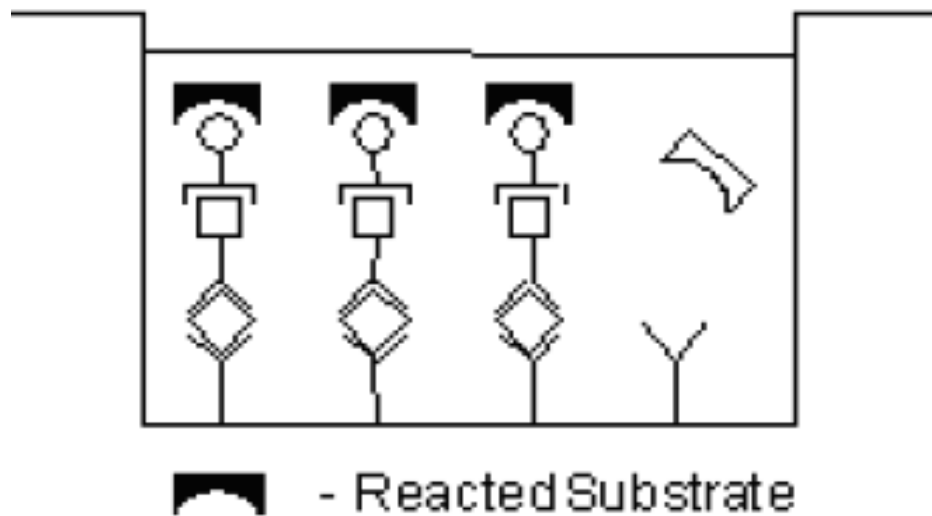


Following incubation unbound biotin conjugated antihuman Leptin and Streptavidin-HRP is removed during a wash step, and substrate solution reactive with HRP is added to the wells.

## Second Incubation



A coloured product is formed in proportion to the amount of soluble human Leptin present in the sample. The reaction is terminated by addition of acid and absorbance is measured at 450 nm. A standard curve is prepared from 7 human Leptin standard dilutions and human Leptin sample concentration determined





### *Reagents Provided*

1 aluminium pouch with a Microwell Plate coated with monoclonal antibody to human Leptin, Biotin-Conjugate (anti-human Leptin monoclonal antibody), Streptavidin-HRP and Assay Buffer, lyophilized

2 aluminium pouches with a human Leptin Standard curve

(coloured)

1 bottle (25 ml) Wash Buffer Concentrate 20x

(phosphate-buffered saline with 1% Tween 20)

1 vial (5 ml) Assay Buffer Concentrate 20x

(Use when an external predilution of the samples is needed)

1 vial (15 ml) Substrate Solution (tetramethyl-benzidine)

1 vial (15 ml) Stop Solution (1M Phosphoric acid)

2 Adhesive Films

### *Storage Instructions*

Store ELISA plate and Standard curves or whole kit at -20°C. The plate and the standard curves can also be removed, stored at -20°C, remaining kit reagents can be stored between 2° and 8°C. Expiry of the kit and reagents is stated on labels. The expiry of the kit components can only be guaranteed if the components are stored properly, and if, in case of repeated use of one

component, the reagent is not contaminated by the first handling.

### *Specimen Collection*

Serum and plasma (EDTA, citrate, heparinized) were tested with this assay. Other biological samples might be suitable for use in the assay. Remove serum or plasma from the clot or cells as soon as possible after clotting and separation.

Samples containing a visible precipitate must be clarified prior to use in the assay. Do not use grossly hemolyzed or lipemic specimens. Samples must be stored frozen at -20°C to avoid loss of bioactive human Leptin. If samples are to be run within 24 hours, they may be stored at 2° to 8°C (for sample stability refer to 13). Avoid repeated freeze-thaw cycles. Prior to assay, the frozen sample should be brought to room temperature slowly and mixed gently.

### *Materials Required But Not Provided*

- ~ 5 ml and 10 ml graduated pipettes
- ~ 5 µl to 1000 µl adjustable single channel micropipettes with disposable tips
- ~ adjustable multichannel micropipettes (for volumes between 50 µl and 500 µl) with disposable tips
- ~ Multichannel micropipette reservoir
- ~ Beakers, flasks, cylinders necessary for preparation of reagents
- ~ Device for delivery of wash solution (multichannel wash bottle or automatic wash system)

- ~ Microwell strip reader capable of reading at 450 nm (620 nm as optional reference wave length)
- ~ Glass-distilled or deionized water
- ~ Statistical calculator with program to perform linear regression analysis

#### *Precautions for Use*

- ~ All chemicals should be considered as potentially hazardous. We therefore recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing such as laboratory overalls, safety glasses and gloves. Care should be taken to avoid contact with skin or eyes. In the case of contact with skin or eyes wash immediately with water. See material safety data sheet(s) and/or safety statements(s) for specific advice.
- ~ Reagents are intended for research use only and are not for use in diagnostic or therapeutic procedures.
- ~ Do not mix or substitute reagents with those from other lots or other sources.
- ~ Do not use kit reagents beyond expiration date on label.
- ~ Do not expose kit reagents to strong light during storage or incubation.
- ~ Do not pipette by mouth.
- ~ Do not eat or smoke in areas where kit reagents or samples are handled.

- ~ Avoid contact of skin or mucous membranes with kit reagents or specimens.
- ~ Rubber or disposable latex gloves should be worn while handling kit reagents or specimens.
- ~ Avoid contact of substrate solution with oxidizing agents and metal.
- ~ Avoid splashing or generation of aerosols.
- ~ In order to avoid microbial contamination or cross-contamination of reagents or specimens which may invalidate the test use disposable pipette tips and/or pipettes.
- ~ Use clean, dedicated reagent trays for dispensing substrate reagent.
- ~ Glass-distilled water or deionized water must be used for reagent preparation.
- ~ Substrate solution must be at room temperature prior to use.
- ~ Decontaminate and dispose specimens and all potentially contaminated materials as they could contain infectious agents. The preferred method of decontamination is autoclaving for a minimum of 1 hour at 121.5°C.
- ~ Liquid wastes not containing acid and neutralized waste may be mixed with sodium hypochlorite in volumes such that the final mixture contains 1.0% sodium hypochlorite. Allow 30 minutes for effective decontamination. Liquid waste containing acid must be neutralized prior to the addition of sodium hypochlorite.

### *Preparation of Reagents and Samples*

Buffer concentrates should be brought to room temperature and diluted before starting the test procedure. If crystals have formed in buffer concentrates, warm them gently until crystals have completely

#### **Wash Buffer (1x)**

Pour entire contents (25 ml) of the Wash Buffer Concentrate (20x) into a clean 500 ml graduated cylinder. Bring to final volume to 500 ml with glass-distilled or deionized water. Mix gently to avoid foaming. Transfer to a clean wash bottle and store at 2° to 25°C. Please note that Wash Buffer (1x) is stable for 30 days.

#### **Assay Buffer**

Pour the entire contents (5ml) of the Assay Buffer Concentrate into a clean 100 ml graduated cylinder. Bring to final volume of 100 ml with distilled water. Mix gently

### *Test Protocol*

- ~ Use plate immediately after removal from -20°C!
- ~ Do not wait until pellets have completely dissolved before applying samples - the binding reaction in the standard strips starts immediately after addition of water!
- ~ Do not try to dissolve pellets by pipetting up and down in the wells - some parts of the pellet could stick to the tip creating high variation of results.

- ~ Perform the washing step with at least 400 µl of washing buffer as stated in the manual or fill the wells completely – otherwise any pellet residues sticking to the rim of the well will not be removed and create high variation of results.
- ~ Allow the washing buffer to sit in the wells for a few seconds before aspiration.
- ~ Remove covers of the standard strips carefully so that all the lyophilized pellets remain in the wells
  - a. Prepare your samples before starting with the test procedure. Dilute serum or plasma samples 1:25 with Assay Buffer according to the following dilution scheme: 10 µl sample + 240 µl Assay Buffer
  - b. Determine the number of Microwell Strips required to test the desired number of samples plus Microwell Strips for blanks and standards (coloured). Each sample, standard and blank should be assayed in duplicate. Remove extra Microwell Strips from holder and store in foil bag with the desiccant provided at -20°C sealed tightly. Place Microwell Strips containing the standard curve in position A1/A2 to H1/H2 (see Table 1).
  - c. Add distilled water to all standard and blank wells as indicated on the label of the standard strips (A1, A2 to H1, H2).
  - d. Add 100 µl of distilled water to the sample wells

Table depicting an example of the arrangement of blanks, standards and samples in the microwell strips:

	<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>
<b>A</b>	Standard 1 (4000 pg/ml)	Standard 1 (4000 pg/ml)	Sample 1	Sample 1
<b>B</b>	Standard 2 (2000 pg/ml)	Standard 2 (2000 pg/ml)	Sample 2	Sample 2
<b>C</b>	Standard 3 (1000 pg/ml)	Standard 3 (1000 pg/ml)	Sample 3	Sample 3
<b>D</b>	Standard 4 (500 pg/ml)	Standard 4 (500 pg/ml)	Sample 4	Sample 4
<b>E</b>	Standard 5 (250 pg/ml)	Standard 5 (250 pg/ml)	Sample 5	Sample 5
<b>F</b>	Standard 6 (125 pg/ml)	Standard 6 (125 pg/ml)	Sample 6	Sample 6
<b>G</b>	Standard 7 (63 pg/ml)	Standard 7 (63 pg/ml)	Sample 7	Sample 7
<b>H</b>	Blank	Blank	Sample 8	Sample 8

e. Add 50 µl of each 1:25 prediluted sample, in duplicate, to the designated wells and mix the contents.

f. Cover with an adhesive film and incubate at room temperature (18°C to 25°C) for 3 hours, if available on a microplate shaker at 400 rpm.

g. Remove adhesive film and empty wells. Wash the microwell strips 6 times with approximately 400 µl Wash Buffer per well with thorough aspiration of microwell contents between washes. Allow the Wash Buffer to sit in the wells for about 10 – 15 seconds before aspiration. Take care not to scratch the surface of the microwells. After the last wash, tap microwell strips on absorbent pad or paper towel to remove excess Wash Buffer. Use the microwell strips immediately after

washing or place upside down on a wet absorbent paper for no longer than 15 minutes. Do not allow wells to dry.

h. Pipette 100  $\mu$ l of TMB Substrate Solution to all wells, including the blank wells.

i. Incubate the microwell strips at room temperature (18° to 25°C) for about 10 min. Avoid direct exposure to intense light. The colour development on the plate should be monitored and the substrate reaction stopped (see next point of this protocol) before positive wells are no longer properly recordable. Determination of the ideal time period for colour development has to be done individually for each assay. It is recommended to add the Stop Solution when the highest standard has developed a dark blue colour. Alternatively the colour development can be monitored by the ELISA reader at 620 nm. The substrate reaction should be stopped as soon as Standard 1 has reached an OD of 0.9 – 0.95.

j. Stop the enzyme reaction by quickly pipetting 100  $\mu$ l of Stop Solution into each well, including the blank wells. It is important that the Stop Solution is spread quickly and uniformly throughout the microwells to completely inactivate the enzyme. Results must be read immediately after the Stop Solution is added or within one hour if the microwell strips are stored at 2 - 8°C in the dark.

k. Read absorbance of each microwell on a spectro-photometer using 450 nm as the primary wave length (optionally 620 nm as the reference wave length; 610 nm to 650 nm is acceptable). Blank the plate reader according to the manufacturer's



instructions by using the blank wells. Determine the absorbance of both the samples and the human Leptin standards.

Note: In case of incubation without shaking the obtained O.D. values may be lower than indicated below. Nevertheless the results are still valid

### **Calculation of Results**

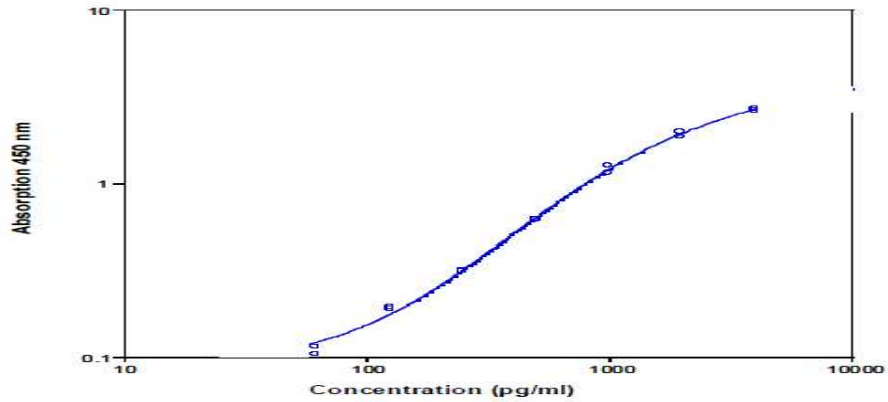
- ~ Calculate the average absorbance values for each set of duplicate standards and samples. Duplicates should be within 20 per cent of the mean.
- ~ Create a standard curve by plotting the mean absorbance for each standard concentration on the ordinate against the human Leptin concentration on the abscissa. Draw a best fit curve through the points of the graph (a 5-parameter curve fit is recommended).
- ~ To determine the concentration of circulating human Leptin for each sample, first find the mean absorbance value on the ordinate and extend a horizontal line to the standard curve. At the point of intersection, extend a vertical line to the abscissa and read the corresponding human Leptin concentration.
- ~ \*Samples have been diluted 1:50, thus the concentration read from the standard curve must be multiplied by the dilution factor (x 50).
- ~ Calculation of samples with a concentration exceeding standard 1 may result in incorrect, low human Leptin levels. Such samples require further external predilution according to expected human Leptin values with Assay Buffer (1x) in order to precisely quantitate the actual human Leptin level.

~ It is suggested that each testing facility establishes a control sample of known human Leptin concentration and runs this additional control with each assay. If the values obtained are not within the expected range of the control, the assay results may be invalid.

~ A representative standard curve is shown in Figure 4. This curve cannot be used to derive test results. Every laboratory must prepare a standard curve for each group of microwell strips assayed.

\* N.B: There is a common dilution factor for samples due to the conjugate which must then be included in the calculation. The samples contribute 100 µl to the final volume per well. These 100 µl are composed of 50 µl of Sample Diluent plus 50 µl of the 1:25 prediluted sample. This is a 1:50 dilution. The remaining 50 µl to give 150 µl are due to the addition of 50 µl conjugate to all wells. 50 µl Sample Diluent and 50 µl conjugate results in 100 µl reconstitution volume, addition of 50 µl 1:25 prediluted sample (50 µl + 50 µl 1:25 prediluted sample = 1:50 dilution).

Representative standard curve for human Leptin Instant ELISA. Human Leptin was diluted in serial 2-fold steps in Assay Buffer. Each symbol represents the mean of 3 parallel titrations. Do not use this standard curve to derive test results. A standard curve must be run for each group of microwell strips assayed.



Typical data using the human Leptin INSTANT ELISA. Measuring wavelength: 450 nm.

Reference wavelength: 620 nm.

Standard	Human Leptin Concentration (pg/ml)	O.D. (450 nm)	O.D. Mean	C.V. (%)
1	4000	2.683 2.622	2.652	1.6
2	2000	1.853 1.968	1.911	4.3
3	1000	1.150 1.257	1.203	6.3
4	500	0.614 0.621	0.618	0.8
5	250	0.310 0.315	0.313	1.1
6	125	0.193 0.189	0.191	1.6
7	63	0.115 0.104	0.109	7.4
Blank	0	0.044 0.050	0.047	9.1

The OD values of the standard curve may vary according to the conditions of assay performance (e.g. operator, pipetting technique, washing technique or temperature

effects). Furthermore shelf life of the kit may affect enzymatic activity and thus colour intensity. Values measured are still valid

### **Limitations**

- ~ Since exact conditions may vary from assay to assay, a standard curve must be established for every run.
- ~ Bacterial or fungal contamination of either screen samples or reagents or cross-contamination between reagents may cause erroneous results.
- ~ Disposable pipette tips, flasks or glassware are preferred, reusable glassware must be washed and thoroughly rinsed of all detergents before use.
- ~ Improper or insufficient washing at any stage of the procedure will result in either false positive or false negative results. Empty wells completely before dispensing fresh wash solution, fill with Wash Buffer as indicated for each wash cycle and do not allow wells to sit uncovered or dry for extended periods.
- ~ The use of radioimmunotherapy has significantly increased the number of patients with human anti-mouse IgG antibodies (HAMA). HAMA may interfere with assays utilizing murine monoclonal antibodies leading to both false positive and false negative results. Serum samples containing antibodies to murine immunoglobulins can still be analysed in such assays when murine immunoglobulins (serum, ascitic fluid, or monoclonal antibodies of irrelevant specificity) are added to the sample

### **Performance Characteristics**

### *Sensitivity*

The limit of detection of human Leptin defined as the analyte concentration resulting in an absorbance significantly higher than that of the dilution medium (mean plus 2 standard deviations) was determined to be 20 pg/ml (mean of 6 independent assays).

### *Reproducibility*

**Intra-assay:** Reproducibility within the assay was evaluated in 3 independent experiments. Each assay was carried out with 6 replicates of 6 serum samples containing different natural levels of human Leptin. 2 standard curves were run on each plate. Data below show the mean human Leptin concentration and the coefficient of variation for each sample (see Table 3). The calculated overall intra-assay coefficient of variation was 5.7%.

The mean human Leptin concentration and the coefficient of variation for each sample:

Positive Sample	Experiment	Human Leptin Concentration (pg/ml)	Coefficient of Variation (%)
1	1	59210	1.9
	2	57051	2.9
	3	57233	1.9
2	1	89608	3.5
	2	88294	2.0
	3	85028	2.1
3	1	23739	11.7
	2	28364	3.5
	3	29049	2.2
4	1	8839	13.6
	2	10774	7.8
	3	10661	7.1
5	1	20568	12.2
	2	23033	4.6
	3	19829	5.3
6	1	8989	10.9
	2	7930	4.9
	3	9120	5.0

**Inter-assay:** Assay to assay reproducibility within one laboratory was evaluated in 3 independent experiments. Each assay was carried out with 6 replicates of 6 serum samples containing different concentrations of human Leptin. 2 standard curves were run on each plate. Data below show the mean human Leptin concentration and the coefficient of variation calculated on 18 determinations of each sample (see Table 4). The calculated overall inter-assay coefficient of variation was 6.9%.

The mean human Leptin concentration and the coefficient of variation of each sample

Sample	Mean human Leptin Concentration (pg/ml)	Coefficient of Variation (%)
1	57832	2.1
2	87643	2.7
3	27051	10.7
4	10091	10.8
5	21143	7.9
6	8680	7.5

**Spike Recovery:** The spike recovery was evaluated by spiking 2 levels of human Leptin into samples. Recoveries were determined in 4 independent experiments with 6 replicates each. The amount of endogenous human Leptin in unspiked samples was subtracted from the spike values. Recovery ranged from 42% to 129% with an overall mean recovery of 86% (see Table 5).

Table 5

Sample matrix*	Spike high (%)	Spike medium (%)
Serum	84	68
Plasma (EDTA)	103	87
Plasma (citrate)	86	105
Plasma (heparin)	81	70

**Dilution Parallelism:** Serum and plasma samples (4 samples of each sample matrix) with different levels of human Leptin were analysed at serial 2 fold dilutions with 4 replicates each. The recovery ranged between 87% and 92% with an overall recovery of 90% (see Table 6).

Table 6

Sample Matrix	Recovery of Exp. Val. (%)
Serum	92
Plasma (EDTA)	87
Plasma (citrate)	97
Plasma (heparin)	91



Table 7 shows detailed recovery data of 4 serum samples.

Table 7

Sample	Dilution	Mean human Leptin Concentration (pg/ml)		% Recovery of Exp. Val.
		Expected Value	Observed Value	
1	1		54504	
	2	27252	25152	92.3
	4	13626	11482	84.3
	8	6813	5449	80.0
2	1		85031	
	2	42516	40833	96.0
	4	21258	19986	94.0
	8	10629	9520	89.6
3	1		28093	
	2	14046	12624	89.9
	4	7023	5626	80.1
	8	3512	2812	80.1
4	1		25307	
	2	12654	11740	92.8
	4	6327	5314	84.0
	8	3164	2857	90.3

### Sample Stability

*Freeze-Thaw Stability:* Aliquots of serum samples (unspiked or spiked) were stored at -20°C and thawed 5 times, and the human Leptin levels determined. There was no significant loss of human Leptin immunoreactivity detected by freezing and thawing

*Storage Stability:* Aliquots of serum samples (spiked or unspiked) were stored at -20°C, 2- 8°C, room temperature (RT) and at 37°C, and the human Leptin level determined after

24 h. There was no significant loss of human Leptin immunoreactivity detected during storage under above conditions

*Specificity:* The assay detects both natural and recombinant human Leptin. The interference of circulating factors of the immune system was evaluated by spiking these proteins at physiologically relevant concentrations into positive serum. There was no crossreactivity detected

### Expected Values

A panel of 40 sera samples from randomly selected apparently healthy donors (males and females) was tested for human Leptin. The detected human Leptin levels ranged between 797 and 508072 pg/ml with a mean level of 12991 pg/ml. The levels measured may vary with the sample collection used. For detected human Leptin levels see Table 8.

Table 8

Sample Matrix	Number of Samples > S7*	Detectable Samples			
		Minimum (pg/ml)	Maximum (pg/ml)	Mean (pg/ml)	Standard Deviation (pg/ml)
Serum	22	1058	45881	9442	10338
Plasma (EDTA)	18	894	50376	14967	15423
Plasma (Citrate)	29	797	38246	9740	10344
Plasma (Heparin)	26	890	58072	17813	15863

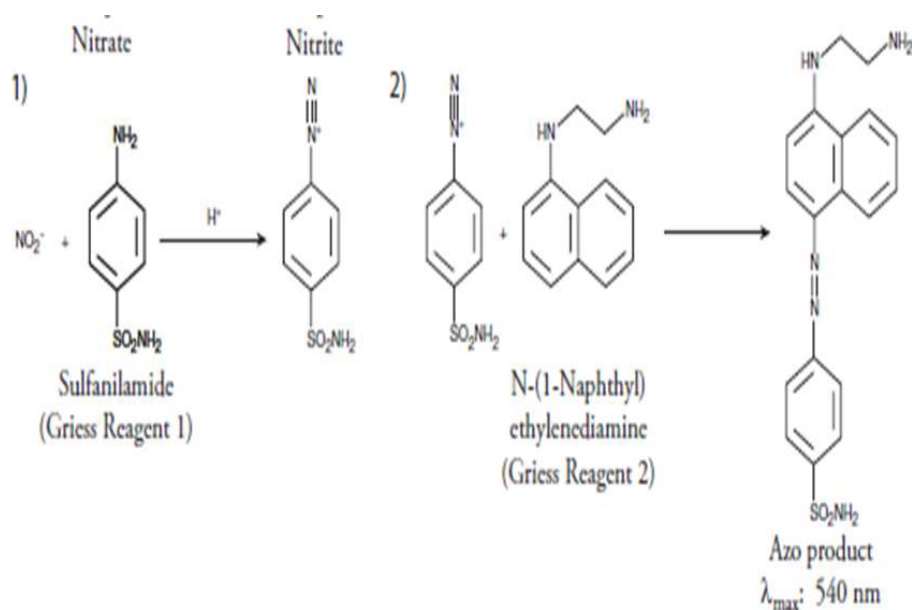
\*S7 = lowest standard point

## APPENDIX B

### NITRIC OXIDE ASSAY PROTOCOL

## ABOUT THIS ASSAY

Caymans nitrate/nitrite colorimetric assay kit provides an accurate and convenient method for measurement of total nitrate/nitrite concentration in a simple two way step process . the first step is the conversion of nitrate to nitrite utilizing nitrate into a deep purple azo compound . photometric measurement of the absorbance due to this azo chromophore accurately determines NO<sub>2</sub> concentration .



NADPH is an essential cofactor for the function of the NOS enzyme.

Unfortunately, NADPH interferes with chemistry of the Griess reagents , which are the most commonly used reagents for nitrite detection . One way to prevent this interference is to use small amounts of NADPH in conjunction with a catalytic system for recycling spent NADP<sup>+</sup> back to NADPH. This is system used in this nitrate/nitrite colorimetric assay kit. It works well for the analysis of nitrate and nitrite in fluids such as plasma and urine , and is also available in a highly sensitive fluorometric version (item No.780051) for the detection of low levels of nitrite . However, it cannot be used to analyze nitrate

and nitrite from an in vitro assay of NOS in which excess NADPH has been added . for these assays a second method (LDH method ) is utilized and is available from Cayman in a 96 well plate format (item 760871).

## **PRE-ASSAY PREPARATION**

### ***1-NITRATE/NITRITE ASSAY BUFFER (ITEM NO. 780022)***

Is obtained by diluting the contents of the assay buffer vial to 100 ml with ultrapure water (milli-Q or equivalent), 1:2 ratio. This assay buffer should be used for dilution of samples as needed prior to assay, the buffer will be stable for approximately two months at 4°C.

### ***2-NITRATE REDUCTASE ENZYME PREPARATION (ITEM NO. 780010)***

Through reconstitute the contents of the vial with 1.2 ml of assay buffer, and keeping it on ice during use. Store at -20°C when not in use. Freezing and thawing of this solution should be limited to one time.

### ***3-NITRATE REDUCTASE COFACTOR PREPARATION (ITEM 780012)***

Thoroughly reconstitute the content of the vial with 1.2 ml of assay buffer. The preparation should be kept on ice during use and store at -20°C when not in use. Freezing and thawing of this solution should be limited to one time

### ***4-NITRATE STANDARD (ITEM NO. 780014)***

Removing the vial stopper slowly to minimize disturbance of the lyophilized powder. Reconstitution of the contents of the vial with 1.0 ml of assay buffer. Vortex and

mix sufficiently to ensure all powder in the vial, including any on the stopper in solution. Storage is at 4°C when not in use. The reconstituted standard will be stable for about four months when stored at 4°C .

***5-NITRITE STANDARD (ITEM NO. 780016)***

By removing the vial stopper slowly to minimize disturbance of the lyophilized powder. Reconstitution the content of the vial with 1.0 ml of assay buffer. Vortex and mix sufficiently to ensure all powder in the vial, including any on the topper. Is in solution. Store at 4°C when not in use. The reconstituted standard will be stable for about four months when stored at 4°C.

***6-GRIESS REAGENTS R1 AND R2 (ITEM NOS. 780018 AND 780020)***

This is without adding any water or assay buffer to these reagents, as they are ready for use. These reagents should be stored at 4°C.

**SAMPLE PREPARATION**

The kit has been validated in urine, culture media and plasma. NO sample purification from these sources is necessary other than some special instruction as described below. Storing of samples should be at -20°C or -80°C after collection.

**1. Urine Samples**

Urine can be used directly after dilution to the proper concentration in assay buffer. Urine contains relatively high levels of nitrate (200-2000 µM), so dilution of approximately 1:10-1:50 may be necessary.

## **2. Culture Media**

Some types of tissue culture media contain very high nitrate levels (RPMI 1640). These types of media should not be used for cell culture if the goal of an experiment is to measure small changes in nitrate levels. Cellular nitrate/nitrite production can be quantitated by subtracting the level of nitrate/nitrite present in the media (in the absence of cells) from the total nitrate/nitrite level present during cell growth. The effect of media components on color development can be assessed by making a nitrite standard curve in the presence of a fixed volume of the culture media (40  $\mu$ l works well ) and comparing it to a nitrite standard curve made in buffer alone.

## **3. Plasma and serum samples**

Ultrafiltration of plasma or serum samples through a 10 or 30 KDa molecular weight cut-off filter. The filters, supplied through Amicon or Millipore, are pre-rinsed with ultra pure water prior ultrafiltration. Ultrafiltration will reduce background absorbance due to the presence of hemoglobin and improve color formation using Griess reagents. Assay for nitrate and / or nitrite using a maximum of 40  $\mu$ l of the filtrate. The conversion of nitrate to nitrite requires three hours for completion.

## **4. Tissue homogenates**

Homogenize the sample in PBS, pH 7.4 and centrifuge at 10000 x g for 20 minutes. Ultracentrifuge the supernatant solution at 100000 x g for 30 minutes (centrifugation at 100000 x g is optional, but will increase filtration rates). Ultrafilter using a 10 or 30 kDa molecular weight cut-off filter using a commercially available centrifuge or microfuge ultrafiltration device. The filters supplied through Amicon or

Millipore, should be pre-rinsed with ultrapure water prior to ultrafiltration. Assay the sample for nitrate and/or nitrite using a maximum of 40  $\mu$ l of the filtrate. The conversion of nitrate to nitrite requires three hours for completion.

## **ASSAY PROTOCOL**

### ***Plate set up***

There is no specific pattern for using the wells on the plate. However it is necessary to have some wells (at least two) designated as absorbance blanks (containing 200  $\mu$ l of assay buffer or water). The absorbance of these wells will be subtracted from the absorbance measured in all the other wells. Standard curves for nitrate and nitrite must also be included. Measuring only total NO products (nitrate + nitrite), only the nitrate standard curve is required. To measure only nitrite, the nitrite standard curve is needed. The wells for the standard curves have been designated (as A1-H2) in the instruction. However, these standard curves can be place in any wells. The remaining wells on the plate can then be used for the assay of samples, it is suggested to record the contents of each well on the template sheets provided .

The kit provides sufficient cofactors and reagents to run two 96 well plates measuring total NO ( $\text{NO}_2 + \text{NO}_3$ ) in all the wells. In case of testing some samples for  $\text{NO}_2$  only (where reductase and cofactor are not required). There is sufficient Griess reagent R1 and R2 to run a third 96 well plate of nitrite determinations. All three plates are supplied with kit.



	1	2	3	4	5	6	7	8	9	10	11	12
A	A	A	S1	S1	S9	S9	S17	S17	S25	S25	S33	S33
B	B	B	S2	S2	S10	S10	S18	S18	S26	S26	S34	S34
C	C	C	S3	S3	S11	S11	S19	S19	S27	S27	S35	S35
D	D	D	S4	S4	S12	S12	S20	S20	S28	S28	S36	S36
E	E	E	S5	S5	S13	S13	S21	S21	S29	S29	S37	S37
F	F	F	S6	S6	S14	S14	S22	S22	S30	S30	S38	S38
G	G	G	S7	S7	S15	S15	S23	S23	S31	S31	S39	S39
H	H	H	S8	S8	S16	S16	S24	S24	S32	S32	S40	S40

A-H = Standards  
S1-S40 = Sample Wells

## MEASUREMENT OF NITRATE + NITRITE

### *PREPARATION OF NITRATE STANDARD CURVE*

A nitrate standard curve must be performed in order to quantitate sample nitrate + nitrite concentration. In a clean test tube place 0.9 of assay buffer and to this we add 0.1 ml of reconstituted nitrate standard and vortex. The concentration of this stock standard is 200  $\mu$ M. We use this standard for the preparation of the nitrate standard curve as described below. The standard curve for nitrate is prepared by addition of reagents to the plate wells in the following way:

Well	Nitrate Standard ( $\mu\text{l}$ )	Assay Buffer ( $\mu\text{l}$ )	Final Nitrate Concentration* ( $\mu\text{M}$ )
A1, A2	0	80	0
B1, B2	5	75	5
C1, C2	10	70	10
D1, D2	15	65	15
E1, E2	20	60	20
F1, F2	25	55	25
G1, G2	30	50	30
H1, H2	35	45	35

***Nitrate standard concentration*** \*the concentration is calculated for the final 200  $\mu\text{l}$  assay volume after addition of the Griess reagents.

#### ***PREPARATION OF SAMPLES FOR TOTAL NITRATE+NITRITE MEASUREMENT***

Samples containing nitrate (with or without nitrite) can be assayed by addition of up to 80  $\mu\text{l}$  (40  $\mu\text{l}$  with plasma or serum) of sample per well and be done in triplicate when using less than 80  $\mu\text{l}$  of sample, the volume is adjusted to 80 $\mu\text{l}$  by addition of the appropriate amount of the assay buffer. When necessary, dilution of samples should be done using the assay buffer solution. In the event that the approximate concentration of nitrate or nitrite is completely unknown, it is recommended that several dilution of the sample is made.

The absorbance of the samples should be between 0.05 and 1.2 absorbance units. Since the plate reader will give the most accurate values when the absorbance is in this range. In addition, high absorbance values imply high nitrate levels. Under these conditions, there may be incomplete conversion of nitrate to nitrite. The detection limit of

the assay is approximately 1  $\mu$ M nitrite. When using 80  $\mu$ l of sample, this translates into 2.5  $\mu$ M nitrate in the original sample.

- It is recommended that an adjustable pipette be used to deliver reagents to the wells.
- Before pipetting each reagent, equilibrate the pipette tip in that reagent
- Do not expose the pipette tip to the reagents already in the well.

### **PERFORMING THE ASSAY**

- 1- Adding 200  $\mu$ l of water or assay buffer to the blank wells, do not add any other reagents to these wells.
- 2- Adding up to 80  $\mu$ l of sample dilution to the wells. The final volume must be adjusted to 80  $\mu$ l using the assay buffer solution. NOTE plasma samples should be assayed with no more than 40  $\mu$ l when undiluted samples are used (samples which have been diluted 1:2 or greater can use up to 80  $\mu$ l in the assay). \*Caution should be taken when pipetting plasma samples to ensure no bubbles enter to the well as this will lead to erroneous results.
- 3- Adding 10  $\mu$ l of the enzyme cofactor mixture to each of the wells.
- 4- Adding 10  $\mu$ l of the Nitrate reductase mixture to each of the wells.
- 5- Cover the plate with the plate cover and incubate at room temperature for one hour and this incubation time should be increased to two hours when assaying tissue culture medium, and increased to three hours when assaying plasma or

- tissue nitrate + nitrite concentration. It is not necessary to shake the plate during incubation.
- 6- After the required incubation time, 50  $\mu$ l of Griess reagent R1 is added to each of the wells.
  - 7- Add immediately 50  $\mu$ l of Griess reagent R2 to each of the wells.
  - 8- Allowing the color to develop for 10 minutes at room temperature, it is not necessary to cover the plate, this 10 minute incubation is optimal for color development however if the plate has been left to develop for longer time periods the data is still valid. Provided the Griess reagents have added to the standard curve at the same time as the unknowns ensures the presence of an accurate control.
  - 9- The absorbance reading is at 550 nm using a plate reader (Thermo scientific multiscan GO).

### ***Calculations***

Subtract the blanks. Average the absorbance value of the blank wells and subtract this from absorbance values of all the other wells.

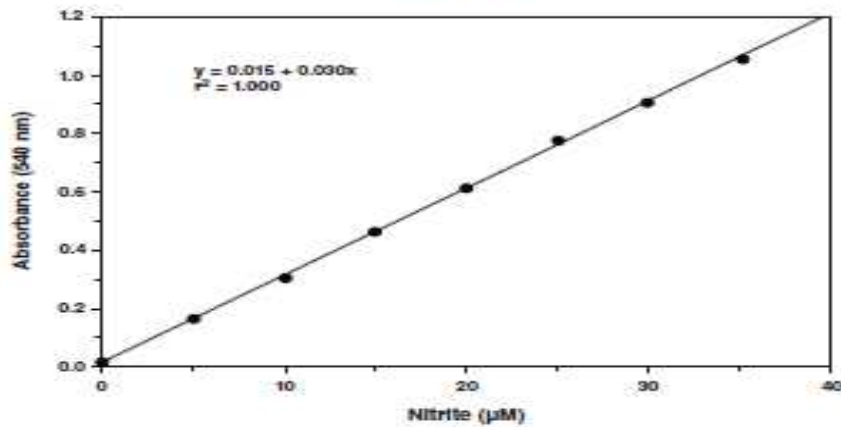
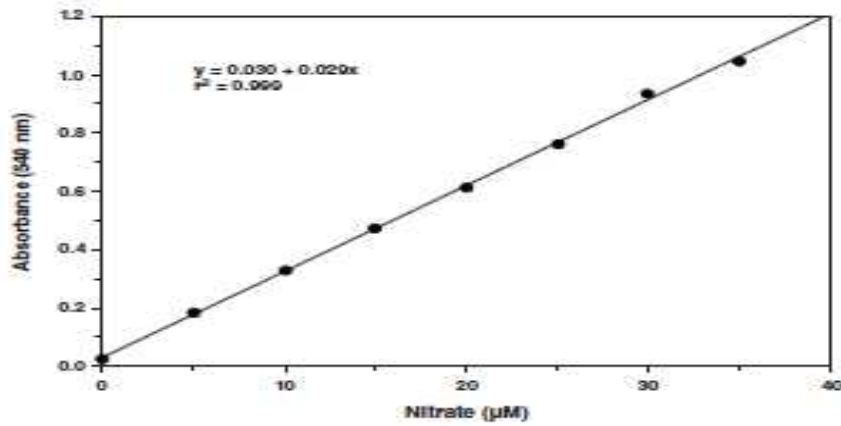
***Plotting the standard curves:*** Make a plot of absorbance at 540-550 nm as a function of nitrite or nitrite concentration. The nitrate standard curve is used for determination of total nitrate + nitrite concentration, whereas the nitrite standard curve is used for determination of nitrite alone. In theory these two standard curves should be identical However, in practice a small discrepancy often occurs according to the kit guide.

***Determination of sample nitrate or nitrite concentration***

$$[\text{Nitrate} + \text{Nitrite}] (\mu\text{M}) = \left( \frac{A_{540} - y\text{-intercept}}{\text{slope}} \right) \left( \frac{200 \mu\text{l}}{\text{volume of sample used} (\mu\text{l})} \right) \times \text{dilution}$$

$$[\text{Nitrite}] (\mu\text{M}) = \left( \frac{A_{540} - y\text{-intercept}}{\text{slope}} \right) \left( \frac{200 \mu\text{l}}{\text{volume of sample used} (\mu\text{l})} \right) \times \text{dilution}$$

$$[\text{Nitrate}] (\mu\text{M}) = (\text{Nitrate} + \text{Nitrite}) - (\text{Nitrite})$$



***Performance characteristics***

Precision: The inter assay coefficient of variation is 3.4%(n=5); The intra assay coefficient of variation is 2.7% (n=84).

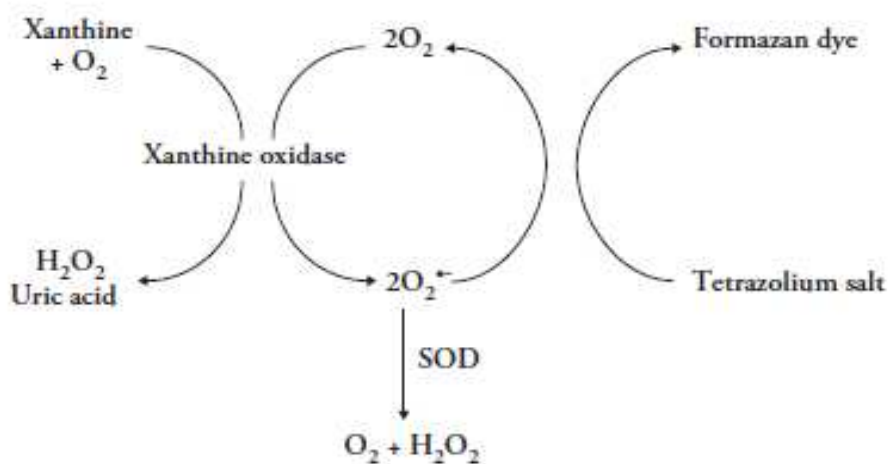
Sensitivity: Using the maximum amount of sample for nitrate/nitrite assay (80  $\mu\text{l}$ ) , the detection limits is 2.5  $\mu\text{M}$  . the detection limit for plasma is higher since only 40  $\mu\text{l}$  of sample can be used . for the nitrite assay a maximum volume of 100  $\mu\text{l}$  can be used . in this case the detection limit is approximately 2.0  $\mu\text{M}$ .

## APPENDIX C

### SUPEROXIDE DISMUTASE ASSAY PROTOCOL

## About this assay

Cayman's superoxide dismutase assay kit utilizes a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. One unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of superoxide radicals. The SOD assay measures all three types of SOD (Cu/Zn, Mn, and FeSOD). The assays provides a simple, reproducible and fast tool for assaying SOD activity in plasma, serum, erythrocyte lysates, tissue homogenates, and cell lysates. Mitochondrial MnSOD can be assayed separately following the procedure outlined under sample preparation.



## Scheme of superoxide dismutase assay

### PRE-ASSAY PREPARATION

#### Reagent preparation

1. Assay buffer (10x)-item No.706001: Dilute 3 ml of Assay Buffer concentration with 27 ml of HPLC-grade water for assaying 96 wells. Prepare additional assay buffer as needed. This final Assay buffer (50 mM Tris-HCL, pH 8.0, containing 0.1 mM



diethylenetriaminepentaacetic acid (DTPA) and 0.1 mM hypoxanthine) will be used to dilute the radical detector. This diluted Assay Buffer is stable for at least 2 months when stored at 4°C.

2. SAMPLE BUFFER (10X)-item No.706003: Dilute 2 ml of sample buffer concentration with 18 ml of HPLC-grade water for assaying 96 wells. Prepare additional sample buffer as needed. This final sample buffer (50 mM Tris-HCL , pH 8.0) will be used to prepare the SOD standards and dilute the xanthine oxidase and SOD samples prior to assaying. This diluted sample buffer is stable for at least 6 months when stored at 4°C.

3. RADICAL DETECTOR (item No 706004): The vials contain 250 µl of tetrazolium salt solution. Prior to use, a 50 µl of the supplied solution is transferred to another vial and diluted with 119.95 ml of diluted assay buffer. Cover with tin foil. The diluted radical detector is stable for 2 hours. This is enough radical detector for 96 wells, the unused radical detector is stored at -20°C.

4. SOD STANDARD – (item No.706005): The vials contain 100µl of bovine erythrocyte SOD (Cu/Zn). Store the thawed enzyme is stored on ice and preparing the standard curve will be discussed in the standard preparation section .the unused enzyme is stored at -20°C. The enzyme is stable for at least two freeze/thaw cycles.

5. XANTHINE OXIDASE – (item No. 706006): These vials contain 150 µl of Xanthine oxidase. Prior to use thaw one vial and transfer 50 µl of the supplied enzyme to another vial and diluting with 1.95 ml of sample buffer (dilute). The thawed and diluted xanthine oxidase is stored on ice. The diluted enzyme is stable for one hour. This is enough

xanthine oxidase for 96 wells. The unused thawed should not be refreeze and should be thrown away.

## **SAMPLE PREPARATION**

The procedures below listed for tissue homogenates and cell lysates will result in assaying total SOD activity (cytosolic and mitochondrial). To separate the two enzymes centrifuge the 1,500 x g supernatant at 10,000 x g for 15 minutes at 4°C. The resulting 10,000 x g supernatant will contain cytosolic SOD and the pellet will contain mitochondrial SOD. Homogenize the mitochondrial pellet in cold buffer (20 mM HEPES , pH 7.2 containing 1 mM EGTA, 210 mM mannitol, and 70 mM sucrose). If not assaying on the same day, freeze the samples at -80°C. The samples will be stable for at least one month. The addition of 1-3 mM of potassium cyanide to the assay will inhibit both Cu/Zn-SOD and extracellular SOD resulting in the detection of only Mn-SOD activity.

Samples can be assayed in the absence of Xanthine oxidase to generate a sample background. This sample background absorbance should be subtracted from the sample absorbance generated in the presence of Xanthine oxidase thus correcting for non SOD generated absorbance.

### **Tissue homogenate**

1. Prior to dissection , either perfuse or rinse with phosphate buffered saline pH 7.4 to remove any red blood cells and clots.
2. Homogenize the tissue in 5-10 ml of cold 20 mM HEPES buffer, pH7.2, containing 1mM EGTA, 210 Mm mannitol, and 70 mM sucrose per gram tissue.

3. Centrifuge at 1,500 x g for five minutes at 4°C.
4. Remove the supernatant for assay and store on ice. If not assaying on the same day freeze the sample at -80°C. The sample will be stable for at least one month.

### **Cell lysate**

1. Collect cells by centrifugation at 1,000-2,000 x g for 10 minutes at 4°C. For adherent cells, do not harvest using proteolytic enzymes, rather use a rubber policeman.
2. Homogenize or sonicate the cell pellet in cold 20 mM HEPES buffer, pH7.2 containing 1 mM EGTA, 210 mM mannitol, and 70 Mm sucrose.
3. Centrifuge at 1,500 x g for five minutes at 4°C.
4. Remove the supernatant for assay and store on ice. If not assaying on the same day, freeze the sample at -80°C. The sample will be stable for at least one month.

### **Plasma and erythrocyte lysate**

1. Collect blood using an anticoagulant such as heparin, citrate or EDTA
2. Centrifuge the blood at 700-1,000 x g for 10 minutes at 4°C. Pipette off the top yellow plasma layer without disturbing the white buffy layer. Store plasma on ice until assaying or freeze at -80°C. The plasma sample will be stable for at least one month .plasma should be diluted 1:5 with sample buffer before assaying for SOD activity
3. Remove the white buffy layer (leukocytes) and discard.

4. Lyse the erythrocytes ( red blood cells ) in four times its volume of ice-cold HPLC-grade water.
5. Centrifuge at 10,000 x g for 15 minutes at 4°C.
6. Collect the supernatant (erythrocyte lysate) for assaying and store on ice. If not assaying the day, freeze at -80°C. The sample will be stable for at least one month. The erythrocyte lysate should be diluted 1:100 with sample buffer before assaying for SOD activity.

### **Serum**

1. Collect blood without using an anticoagulant such as heparin, citrate or EDTA, allow blood to clot for 30 minutes at 25°C.
2. Centrifuge the blood at 2,000 x g for 15 minutes at 4°C. Pipette off the top yellow serum layer without disturbing the white buffy layer. Store serum on ice. If not assaying the same day, freeze at -80°C. The sample will be stable for at least one month.
3. Serum should be diluted 1:5 with sample buffer before assaying for SOD activity.

### **ASSAY PROTOCOL**

#### ***Plate set up***

There is no specific pattern for using the wells on the plate. A typical layout of SOD standards and samples to be measured in duplicate is given below in the figure:

	1	2	3	4	5	6	7	8	9	10	11	12
A	A	A	S2	S2	S10	S10	S18	S18	S26	S26	S34	S34
B	B	B	S3	S3	S11	S11	S19	S19	S27	S27	S35	S35
C	C	C	S4	S4	S12	S12	S20	S20	S28	S28	S36	S36
D	D	D	S5	S5	S13	S13	S21	S21	S29	S29	S37	S37
E	E	E	S6	S6	S14	S14	S22	S22	S30	S30	S38	S38
F	F	F	S7	S7	S15	S15	S23	S23	S31	S31	S39	S39
G	G	G	S8	S8	S16	S16	S24	S24	S32	S32	S40	S40
H	S1	S1	S9	S9	S17	S17	S25	S25	S33	S33	S41	S41

A-G = Standards

S1-S41 = Sample Wells

- It is recommended that an adjustable pipette be used to deliver reagents to the wells.
- Before pipetting each reagent, equilibration is done to the pipette tip in that reagent (slowly filling the tip and gently expel the contents, repeating several times).
- Do not expose the pipette tip to the reagents already in the well.

**General information**

- The final volume of the assay is 230  $\mu$ l in all the wells.
- The assay temperature is 25°C.
- All reagents except samples and xanthine oxidase must be equilibrated to room temperature before beginning the assay.

- It is recommended that samples and SOD standards be assayed at least in duplicate.
- The absorbance monitoring is at 440-460 nm using a plate reader.

## STANDARD PREPARATION

Diluting 20  $\mu\text{l}$  of the SOD standard (item No.706005) with 1.98 ml of sample buffer (dilute) to obtain the SOD stock solution. Take seven clean glass test tubes and mark them A-G and adding the amount of SOD stock and sample buffer to each tube as showing in the table.

Tube	SOD Stock ( $\mu\text{l}$ )	Sample Buffer ( $\mu\text{l}$ )	Final SOD Activity (U/ml)
A	0	1,000	0
B	20	980	0.025
C	40	960	0.05
D	80	920	0.1
E	120	880	0.15
F	160	840	0.2
G	200	800	0.25

## SUPEROXIDE DISMUTASE STANDARDS

### Performing the assay

1. SOD standards wells – through adding 200  $\mu\text{l}$  of the diluted radical detector and 10  $\mu\text{l}$  of standard tubes A-G per well in the designated wells on the plate.
2. Sample wells – through adding 200  $\mu\text{l}$  of the diluted radical detector and 10  $\mu\text{l}$  detector and 10  $\mu\text{l}$  of sample to the wells.

3. Initiating the reactions by adding 20  $\mu$ l of diluted xanthine oxidase to all the wells using. It is important to precise the time of starting adding the xanthine oxidase and as quickly as possible.
4. Shaking the 96 well plate for few seconds to mix and covering with the plate cover.
5. Incubation of the plate on a shaker for 20 minutes and reading the absorbance at 440-460 nm using a plate reader (Thermo Scientific Multiscan GO).

## **Analysis**

### **Calculations**

1. Through calculation the average absorbance of each standard and sample and subtracting sample background absorbance from sample.
2. Dividing standard A's absorbance by itself and divide standard A's absorbance by all the other standards and samples absorbance to yield the linearized rate (LR) which is stands for  $\text{Std A} = \text{Abs Std A} / \text{Abs Std A}$ ; LR for  $\text{Std B} = \text{Abs Std A} / \text{Abs Std B}$ .
3. We plotted the linearized SOD standard rat (LR) as a function of jinal SOD activity (U/ml) for a typical standard curve.
4. The calculation of the SOD activity of the samples by using the equation obtained from the linear regression of the standard curve substituting the linearized rate (LR) for each sample. One unit is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. SOD

activity is standardized using the cytochrome c and xanthine oxidase coupled assay.

$$\text{SOD (U/ml)} = \left[ \left( \frac{\text{sample LR} - \text{y-intercept}}{\text{slope}} \right) \times \frac{0.23 \text{ ml}}{0.01 \text{ ml}} \right] \times \text{sample dilution}$$

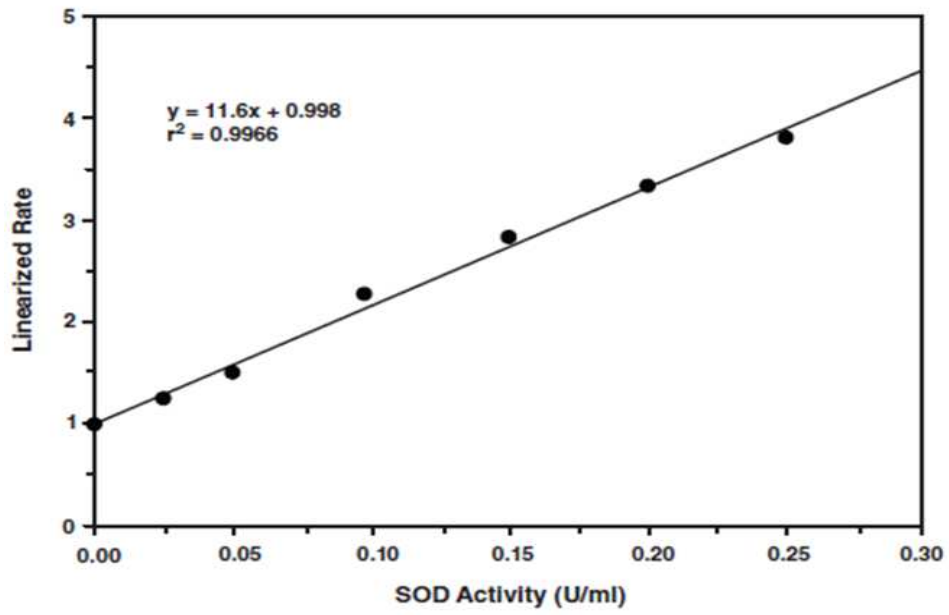
### **Performance characteristics**

**Precision:** When a series of 60 SOD standard measurement were performed on the same day, the intra assay coefficient of variation was 3.2% and when a series of 60 SOD standard measurements were performed on five different days under the same experimental conditions, the inter assay coefficient of variation was 3.7%.

**Assay range:** Under the standardized conditions of the assay described , the dynamic range of the kit is 0.025-0.25 units /ml SOD.

**Representative superoxide dismutase standard curve:** The standard curve presented here is an example of the data typically provided with this kit.





Superoxide dismutase standard curve

APPENDIX D

ABBREVIATIONS

AgRP	agouti-related protein
BMR	basal metabolic rate
CYP450	cytochrome P450
CVD	cardiovascular disease
CCK	cholecystokinin
CCF	cerebral spinal fluid
COX-2	cyclooxygenase-2
e NOS	endothelial nitric oxide synthase
EDRFs	endothelial-derived relaxing factors
GCSF	granulocyte-colony stimulating factor
GPCR	G protein coupled receptors
GLP-1	glucagon like peptide-1
GPR120	G-protein coupled receptors
i NOS	inducible nitric oxide synthase
ICAM-1	intercellular cell-adhesion molecule-1
IL-6	interleukin 6
INF- $\gamma$	interferon- $\gamma$
JAK	janus kinase enzyme
LDL	low density lipoprotein
LIF	leukemia inhibitory factor
MEGJs	myoendothelial gap junctions
MCH	melanin concentrating hormone
NO	nitric oxide
NPY	neuropeptide Y
NF $\kappa$ B	kappa B nuclear transcription factor

n NOS	neural nitric oxide synthase
PPAR	peroxisome proliferator-activated receptor
POMC	pro-opiomelanocortin
PTPN 11	tyrosine-protein phosphatase non-receptor type 11
PI3K	phosphatidylinositol 3-kinase
PDE3B	phosphodiesterase 3B, cGMP-inhibited
POMC	proopiomelanocortin
RANKL	receptor activator of nuclear factor kappa-B ligand
ROC3	receptor-operated channels
RNS	reactive nitrogen species
RXR $\beta$	retinoid-activated nuclear receptor
STAT	signal transducer and activator of transcription
SOCS3	suppressor of cytokine signaling-3
SOC3	store-operated channels
TNF	tumor necrosis factor
VCAM-1	vascular cell adhesion molecule-1