DIET, ADIPOSITY, AND ADVANCED GLYCATION END PRODUCTS

A DISSERTATION

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

IN THE GRADUATE SCHOOL OF THE

TEXAS WOMAN'S UNIVERSITY

DEPARTMENT OF NUTRITION AND FOOD SCIENCES

COLLEGE OF HEALTH SCIENCES

BY

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DENTON, TEXAS

MAY 2013

DEDICATION

For my husband, Johnny, and my children Eli and Isaac for making life worth living!

ACKNOWLEDGMENTS

I wish to acknowledge the professors, advisors, and colleagues who have supported me and advised me throughout my time at Texas Woman's University.

From Dr. Imrhan I have learned the importance of precision, being detail-oriented in keeping track of one's research materials, and having an organized research agenda.

From Dr. Vijay I have learned that research is best broken down to its component parts, sorted carefully, and then re-assembled prior to careful execution.

From Dr. Prasad I have learned to consider a publisher's point of view, to find what is interesting in my results, and present those facts in the most visually interesting way possible.

From Dr. Mills I have learned that meticulous attention to the theory, conceptualization and method behind each assay is essential to proper execution.

From Drs. Juma and Mo I have learned how to carefully and thoughtfully construct a grant proposal and how to present one for an audience outside my own discipline.

From all of these and from Drs. Landdeck, Longley, DiMarco, Bednar, and Warren I have obtained moral support and the boost I need to keep going when times get rough.

Most importantly, I acknowledge my family whose support and love has made my further education possible and tolerable.

ABSTRACT

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MAY 2013

The purpose of this dissertation is to explore factors which may affect the serum soluble receptor for advanced glycation end products (sRAGE) and to determine whether macronutrient diet composition impacts inflammation and levels of serum n-epsilon-carboxymethyl-lysine (CML), a measure of advanced glycation endproducts.

In study I, we tested the hypothesis that sRAGE is correlated to adiposity in a young, ethnically-diverse population. Seventy-two healthy adult participants were recruited for the study and anthropometric measurements were collected. Sera from all participants were analyzed for sRAGE and CML. In addition, in a subgroup of participants (n=34), serum levels of adiponectin, total cholesterol, high density lipoprotein, and triacylglycerol were determined. sRAGE was inversely correlated with weight (-0.364; p=0.002), waist circumference (-0.334; p=0.004), and BMI (-0.364; p= 0.002). High molecular adiponectin was positively correlated to sRAGE (0.416; p=0.02). This is the first time these associations have been found in a young, ethnically-diverse population.

In study II, we hypothesized that dietary fat content influenced serum sRAGE, CML, and markers of inflammation. Eighteen subjects participated in a feeding study of two diets, one high in fat and the other low in fat, both high in dietary AGEs. Participants were provided breakfast, lunch, dinner and snacks for a single day. Baseline and postprandial sera were analyzed for CML,

sRAGE, adiponectin and C-reactive protein (CRP). Compared to high-fat diet, low-fat diet decreased serum CML.

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CHAPTER I

INTRODUCTION

Advanced glycation end products (AGE) are a diverse group of compounds formed when glucose or other reducing sugars react with amino acids, nucleotide bases or fatty acids, forming glycosylated molecules. Some of these products include pentosidine, carboyxmethyllysine (CML), hydroimidazolone, furosine, and glucosepane (Semba 2010). Those AGEs formed from reactions with polyunsaturated fatty acids are known as ALEs (advanced lipoperoxidation end products) (Kalousova 2005; Uribarri 2005; Semba 2010). AGEs are produced when the Amadori product of the interaction between a reducing sugar and the amino acid attached to a protein, lipid or nucleic acid is further modified by dehydration, oxidation, rearrangement or other reactions, leading to the formation of cross linked derivatives of sugar and other molecules (Barlovic 2011).

Current research suggests that AGEs have both endogenous and exogenous (dietary) origins and may increase oxidative and carbonyl stress by interacting with the receptor for AGE (RAGE), decreasing cardiovascular endothelial dysfunction and exacerbating the consequences of diabetes. The soluble receptor for AGE (sRAGE) is thought to act as a decoy receptor, aiding the body in ridding it of AGEs.

Endogenous AGE formation is up-regulated in conditions of metabolic stress such as sepsis and insulin resistance and in disease states such as diabetes and chronic kidney disease. Endogenous AGE production generally occurs slowly; however, conditions such as those listed above increase oxidative stress and thus may increase formation of AGE (Barlovic 2011). Hyperglycemia may lead to up-regulation of pathways other than glycolysis including the polyol

pathway, which increases AGE production. Activation of protein kinase C could also stimulate the hexosamine pathway, increasing endogenous AGE. Furthermore, an increase in reactive dicarbonyls and reduced detoxification by the glyoxalase system is thought to lead to a state of carbonyl stress, which may increase endogenous AGE (Barlovic 2011).

The body has some capacity to degrade AGEs. Degradative enzymes including glyoxalase I and II help to degrade and detoxify AGE along with aldose reductase and carbonyl reductase, which reduce AGEs, decreasing their destructive potential. Circulating proteins such as lysozyme, defensins and lactoferrin may also bind AGEs in circulation, preventing them from binding elsewhere within the cells (Vlassara 2008). However, it appears that degradation is not always rapid enough to keep up with production. In cases of hyperglycemia or severe oxidative stress, degradation may not keep up with production. In fact, in kidney disease the body appears to decrease its capacity to degrade and excrete AGE to sufficient levels (Koschinsky 1997).

There are two types of cell surface AGE receptors (RAGE/AGER), one which binds AGE and initiates cell activation and another which binds, internalizes and degrades AGE. The membrane bound RAGE receptor for AGE is thought to initiate oxidative stress whereas the AGERs 1, 3, and CD35 receptor help to mediate degradation of AGE (Vlassara 2008). Damage caused by AGEs is caused by activation of nuclear factor kappa beta (NF-κβ) which leads to up-regulation of genes for cytokines, growth factors and cell adhesion molecules (Kalousova 2005). In addition sRAGE, a truncated variant of the parent receptor, may play an anti-inflammatory role by binding to AGE, making it unavailable for RAGE.

Because AGEs are essentially glycated molecules, one might expect that foods rich in carbohydrates would be important dietary sources. This is generally not the case. Exogenous AGE consumption typically increases in high fat diets and when high temperature cooking

methods such as deep-frying, broiling, roasting and grilling are used. High protein products cooked in these ways tend to be particularly high in AGEs (Semba 2010).

Whether endogenous AGE is primarily significant or whether exogenous AGE may contribute significantly to deleterious health effects is a topic under current research. Exogenous AGE or dietary AGEs were once considered insignificant; however, this viewpoint is changing. Early research showed that only 10% of dietary AGE is absorbed (Sgarbieri 1973). Another study evaluating absorption in 43 diabetic subjects and five non-diabetic subjects was completed in 1997 with similar results: about 10% of dietary AGE was found to be absorbed with just 30% being excreted in persons with normal kidney function and as little as five percent excreted in those with impaired kidney function (Koschinsky 1997).

It has been reported that Western diet is high in AGE, but studies which clearly document a controlled, repeatable method of determining AGE in foods and capturing AGE concentrations in a variety of foods and diets are lacking. In most studies, CML has been the AGE used to determine AGE content of foods (Semba 2010). Many later studies have used the tables developed by one particular group to calculate AGE in their intervention studies rather than measuring AGE in the test foods (Goldberg 2004). The Goldberg study took a variety of foods purchased from convenience stores and fast food restaurants and prepared them using standard cooking times with variation in cooking methods: boiled, broiled, deep fried, oven fried and roasted. All foods were analyzed for AGE using an analysis that quantifies CML using ELISA. However, the methods section does not detail whether multiple samples/trials were done in order to determine AGE, a possible limitation of the research method. This study found that AGE was lowest in carbohydrate foods with the lowest within this group being found in milk, followed by vegetables and fruits. Broiled beef, and chicken, oils heated to high levels and roasted nuts were

among the highest CML foods. Using these charts the researchers analyzed 3 day food records from healthy participants and found mean daily AGE intake to be about 16,000 +/- 5000 kU AGE. Persons with diabetes were found to have higher AGE intake.

The impact of dietary AGE on inflammatory markers has been assessed in at least one study. Uribarri et. al. correlated calculated dietary AGE content and serum AGE in 90 healthy subjects finding increases in CRP with both dietary AGE and serum AGE (Uribarri 2005). In five participants taken from this group of 90, short term dietary AGE restriction decreased AGE levels by 30-40 percent. Thus it seems likely that dietary AGE may significantly impact AGE levels even in healthy individuals despite somewhat low absorption. Further, restriction of dietary AGE may decrease inflammatory markers.

OPPORTUNITIES IN RESEARCH

Adiposity has been linked to increased AGEs in some studies and to decreased sRAGE in older, ethnically homogenous populations. How adiposity relates to AGE and sRAGE in a young, diverse population has not been described.

Another area of research yet to be fully elucidated in the field of AGE/RAGE biology and nutrition includes how dietary AGE and other factors may influence disease risk. A more thorough review of the literature will help to elucidate these factors.

Finally, within the field of AGE/RAGE biology and nutrition knowledge of the interaction of dietary AGE and sRAGE is incomplete. While the literature documents many characteristics which may affect sRAGE (BMI, TG, sex, age), it is not well established whether a dietary challenge which is high in AGE may modify sRAGE levels. Even the role of dietary AGE in determining serum AGE is now in question since the publication of a study by Semba et. al. in

early 2012 which found no relationship between dietary records detailing AGE intake and serum AGE.

It is not clear whether dietary macronutrient composition impacts the effect of a high AGE diet. Studies have compared a high AGE low-fat diet to a low AGE low-fat diet; however, no study compares high AGE low fat and high AGE high fat diets. Much has been made of the importance of low fat diet in lowering risk of cardiovascular disease. However, cooking methods recommended for lowering fat intake such as broiling, grilling and roasting tend to increase concentrations of advanced glycation end products (AGEs) in foods. Current research suggests that AGEs increase oxidative and carbonyl stress, may increase cardiovascular endothelial dysfunction, and contribute to cellular damage related to diabetes and chronic kidney disease. In addition increased body fat and body weight are associated with higher levels of AGE and decreased levels of an important receptor for AGE (sRAGE). Since low fat diet is often recommended for weight control in obese and overweight persons, it is important to determine whether a low fat diet prepared using these commonly advised cooking methods contributes to AGE load and negatively affects inflammatory cytokines thought to be influenced by AGE.

Will a low fat diet cooked using these high heat methods have equivalent effects on serum AGE and inflammatory cytokines when compared to a high fat diet cooked in these ways? Will consumption of a low fat diet using these cooking methods have the same effects on serum AGE and sRAGE compared to a high fat diet?

OBJECTIVES

There are three parts to this study. The purpose of the first part is to describe the relationship among indicators of adiposity, CML, sRAGE, and other metabolic biomarkers in young, healthy, ethnically-diverse adults.

The purpose of the second part is to summarize and describe the relationship among dietary AGE and disease risk in adults.

The purpose of the third part is to determine the effect of a high AGE challenge meal on serum AGE, sRAGE and indicators of inflammation including C-reactive protein (CRP) and high molecular weight (HMW) adiponectin in overweight/obese, healthy individuals ages 20-45. This study will also evaluate the effect of both a low-fat high AGE and high-fat high AGE diet on these indicators. In addition because recent studies have shown no correlation between dietary records detailing AGE dietary content and serum AGE, the study will help elucidate whether dietary AGE is important in determining serum AGE.

CENTRAL HYPOTHESES

- sRAGE will correlate inversely with indicators of adiposity in young, healthy, ethnically diverse adults.
- sRAGE will increase in response to an AGE bolus.
- AGE will increase in the serum of participants in response to an AGE bolus and will remain elevated the next day.
- Participants consuming high AGE diets will have increases in serum levels of AGE (measured using CML), increased levels of sRAGE and increased levels of CRP.
- Participants consuming the low-fat high AGE diet will have a reduced response in serum AGE (CML), sRAGE and CRP compared to those consuming the high fat high AGE diet.

CHAPTER II

REVIEW OF LITERATURE

ADVANCED GLYCATION END PRODUCTS (AGEs)

Significance in Physiology

Advanced glycation end products (AGE) are a diverse group of compounds formed when glucose or other reducing sugars react with amino acids, nucleotide bases or fatty acids, forming glycosylated products. Some of these products include pentosidine, carboyxmethyllysine (CML), furosine, hydroimidazolone and glucosepane (Semba 2010). In order for AGEs to be produced, the Maillard browning reaction must first occur. This non-enzymatic reaction takes place when a reducing sugar interacts with amino groups attached to proteins, lipids or nucleic acids, producing a Schiff base which then forms more stable Amadori products. Up to the point of the creation of Amadori products, these reactions are reversible, so AGEs may *not* result from the initial browning reaction. AGEs are produced when the Amadori products are further modified by dehydration, oxidation, rearrangement or other reactions, leading to the formation of cross linked derivatives of sugar and other molecules (Barlovic 2011). Two well known Amadori products are hemoglobin A1c and fructosamine; however, neither of these are AGEs.

AGEs have both endogenous and exogenous (dietary) origins and are thought to contribute to oxidative stress within the body (Vlassara 2002). Endogenous AGE formation is up-regulated in conditions of metabolic stress such as sepsis and insulin resistance and in disease states such as diabetes and chronic kidney disease. Endogenous AGE production generally occurs slowly; however, conditions such as those listed above increase oxidative stress and thus may

increase formation of AGEs (Barlovic 2011; Vlassara 2002). Hyperglycemia may lead to up-regulation of pathways other than glycolysis including the polyol pathway, which increases AGE production. Activation of protein kinase C could also stimulate the hexosamine pathway, increasing endogenous AGE. Furthermore, an increase in reactive dicarbonyls and reduced detoxification by the glyoxalase system is thought to lead to a state of carbonyl stress, which may increase endogenous AGE (Barlovic 2011).

Degradative enzymes including glyoxalase I and II help to degrade and detoxify AGE along with aldose reductase and carbonyl reductase, decreasing their destructive potential. Circulating proteins such as lysozyme, defensins and lactoferrin may also bind AGEs in circulation, preventing them from binding elsewhere within the cells (Vlassara 2008; Vlassara 2002). However, it appears that degradation is not always rapid enough to keep up with production. In cases of hyperglycemia or severe oxidative stress, degradation may not keep up with production. In kidney disease the body appears to decrease its capacity to degrade and excrete AGE to sufficient levels (Koschinsky 1997).

There are two types of cell surface AGE receptors, one which binds AGE and initiates cell activation and another which binds, internalizes and degrades AGE. The RAGE receptor for AGE is thought to initiate oxidative stress whereas the AGERs 1, 3, and CD35 receptors help to mediate degradation of AGE (Vlassara 2008). Damage caused by AGEs is caused by activation of nuclear factor kappa beta (NF-κβ) which leads to up-regulation of genes for cytokines, growth factors and cell adhesion molecules (Kalousova 2005).

Dietary AGEs: Current Dietary Data

Because AGEs are essentially glycated molecules, one might expect that foods rich in carbohydrates would be important dietary sources. Instead exogenous AGE consumption

typically increases in high fat diets and when high temperature cooking methods such as deep-frying, broiling, roasting and grilling are used. This is particularly true for high protein products (Semba 2010; Uribarri 2010). In addition, various cooking techniques may inhibit AGE production including exposing meats to acidic solutions (marinating) and chemical AGE inhibitors (aminoguanidine) (Uribarri, 2010).

Whether endogenous AGE is primarily significant or whether exogenous AGE may contribute significantly to deleterious health effects is a topic under current research. Exogenous AGE or dietary AGEs were once considered insignificant; however, this viewpoint is changing. Early research showed that 10% of dietary AGE is absorbed (Sgarbieri 1973). Another study evaluating absorption in 43 diabetic subjects and five non-diabetic subjects completed in 1997 resulted in similar findings: about 10% of dietary AGEs were found to be absorbed with just 30% being excreted in persons with normal kidney function and as little as five percent excreted in those with impaired kidney function (Koschinsky 1997). An *in vitro* experimental model assessing diet derived AGEs' effects on glutathione (GSH) and GSH peroxidase (GPx), indicators of oxidative stress, on human umbilical vein endothelial cells (HUVECs), determined that AGEs depleted GSH and increased GPx activity (Cai 2002). Thus, it was concluded that food-derived AGEs, prior to absorption at least, do contain carbonyl species that may induce oxidative damage.

It has been reported that Western diet is high in AGEs. At least two large studies have attempted to quantify AGEs in a variety of foods (Goldberg 2004; Uribarri, 2010). These studies were performed by the same group using the same method (ELISA with anti-CML antibody). Another smaller study by Hull et. al. used HPLC-mass spectrophotometry to determine CML concentrations in foods (Hull, 2012). In most studies, CML has been the AGE used to determine AGE content of foods (Semba 2010). This is based on earlier studies indicating that CML levels

directly correlate with levels of other protein or lipid AGEs (Cai 2008; Brownlee 2001; Requena 1997). Thus, while information on AGEs in foods is increasing, studies capturing a variety of AGE concentrations in a variety of foods and diets are lacking. In addition, methods used to determine AGEs in foods vary, and the method used to calculate AGEs in the largest studies (Goldberg 2004 and Uribarri 2010) have been criticized for the lack of specificity of the method and for using "units per 100 g" rather than a more specific quantification.

Many recent dietary intervention studies have used the tables developed by one particular group to calculate AGE in their test diets rather than measuring AGE in the test foods (Goldberg 2004). The Goldberg study took a variety of foods purchased from convenience stores and fast food restaurants and prepared them using standard cooking times with variation in cooking methods: boiled, broiled, deep fried, oven fried and roasted. All foods were analyzed for AGE using an analysis that quantifies CML using ELISA. However, the methods section does not detail whether multiple samples/trials were performed in order to determine AGE, a possible limitation of the research method. Using this method of determining AGE, AGEs were lowest in carbohydrate foods with the lowest levels within this group being found in milk, followed by vegetables and fruits. Broiled beef, and chicken, oils heated to high levels and roasted nuts were among the highest CML foods. Using these charts the researchers analyzed 3 day food records from healthy participants and found mean daily AGE intake to be about 16,000 +/- 5000 kU AGE. Persons with diabetes were found to have higher AGE intake.

A follow-up study by the same group repeated this same process but using greater variations in cooking techniques: marinating, various temperatures, AGE inhibitors, and made new discoveries in this area of research (Uribarri, 2010). According to this research fats have higher levels of AGEs per gram, but meats contribute more to dietary AGEs due to larger serving

sizes. Beef and cheese were found to have the highest levels of AGEs followed by poultry, pork, fish and eggs. These are interesting findings given the fact that red meats have come to be synonymous with heart disease in the public mind. In addition, higher fat and aged cheeses were found to have more AGEs than lower fat cheeses. Methylglyoxal levels (MG) were also quantified in this study and found to correlate well with CML; however, it is clear from reviewing the tables in the article that for some foods, concentrations of CML and MG are very different.

The study by Hull et. al. which used HPLC-MS to determine CML in foods actually found that AGEs were highest in cereal and lowest in fruits and vegetables expressed as mg/100 g of food. However, CML remained highest for meat products when expressed as mg per average serving. (Hull, 2012).

A brief discussion of several studies which have quantified AGE in foods follows. A study of infant formulas purchased in Europe found elevated concentrations of various AGEs including CML with 3-8 fold higher concentrations in liquid infant formula compared to cow's milk and 2.5-5 times higher concentration in powdered infant formula compared to standard powdered milk (Birlouez-Aragon 2004). In addition, a study comparing concentration of AGE in breast milk and formula fed infants found infant formula to be 70 times higher in CML compared to breast milk. Plasma levels of AGE were also consistently higher AGE in formula fed infants compared to breast-fed infants (Sebekova 2008).

A study of a variety of common foods published in 2009 found levels of CML to vary from as low as 0.3 mg/kg of raw milk and 0.35 mg/kg of skim milk to as high as 46.1 mg/kg of wholemeal bread crust (compared to just 4.45 mg/kg of bread crumb) (Assar 2009). Commercial breakfast cereals, ice cream and barbecue sauces also appear to be sources of AGE (Delgado-Andrade 2006; Drusch 1999; Chao 2009). An evaluation of a variety of processing methods for

nuts and seeds found that CML production was increased by roasting methods. Thus consumption of cooked foods compared to raw foods increases AGE ingestion (Yaacoub 2008).

Low-fat vegan diets are lower in AGEs (McCarty 2004). However, this may not be a benefit of vegan diet. In fact, some research indicates that plasma AGE is actually higher in vegans despite their low ingestion of high AGE foods, perhaps due to dietary interaction between low taurine ingestion combined with low AGE intake.

Intermediate products which may or may not form AGEs include methylglyoxl (MG) and glyoxal (GO). Concentrations of these compounds have also been evaluated in foods. In one study a variety of commonly found soft drinks were analyzed. In general the trend was for products containing high fructose corn syrup to be relatively high in MG whereas diet drinks were not (Tan 2008). However, a study by Uribarri et. al. which quantified both CML and MG found high quantities of MG in diet Coke and low MG in regular Coke with low quantities of CML in both diet and regular Coke. For Pepsi, the reverse was true; regular Pepsi had high levels of MG whereas some forms of diet Pepsi had low levels of MG, and both had low levels of CML (Uribarri 2010).

In yet another analysis in which soft drinks were evaluated for AGEs using ELISA, AGE levels in soft drinks did not correlate with sugar content as long as protein and heat were not present. In fact drinks with caramel additives such as Coke Classic or Diet Coke were found to contain 8500 and 9500 units/cup compared to 475 units per cup in Sprite, 600 in orange juice and around 2000 in coffee and tea. (Koschinsky 1997).

One problem with these studies is that they vary in the method of quantifying AGE, and some quantify CML versus other AGE products. Some use ELISA, others GC and others HPLC. Methods of extraction also vary for analyses. Thus, having some standard methods for

determining and quantifying AGE might help in creating comparable studies (Ames 2008; Delgado-Andrade 2007; Drusch 1999). The techniques used by opposing groups have been published and repeated (Hull 2012; Ames 2008; Assar 2009; Uribarri 2010; Goldberg 2004; Cai 2002; Mitsuhashi 1997), but given different units of measurement and different findings, the data remains confusing.

Potential Negative Consequences of AGE Consumption

Current studies have many limitations, yet some trends appear. Uribarri et. al. correlated calculated dietary AGE content and serum AGE in 90 healthy subjects finding increases in CRP to be correlated with both dietary AGE and serum AGE (Uribarri 2005). In five participants taken from this group of 90, short term dietary AGE restriction decreased AGE levels by 30-40 percent. Another study of healthy subjects randomized to a low AGE diet also found reductions in 8-isoprostanes, vascular cell adhesion protein 1 (VCAM-1) and tumor necrosis factor-α (TNF-α) in subjects following the diet (Vlassara, 2009). In studies of mice assigned to high fat high AGE or high fat low AGE diets, similar trends were identified, with mice in the high fat high AGE group accumulating more visceral fat, the majority of them developing diabetes and having higher body weight and serum AGES than the high fat low AGE fed mice or the controls (Sandu 2005). In addition, the high fat high AGE diet fed mice had higher levels of 8-isoprostanes and lower adiponectin. A separate study of 36 diabetic and 18 healthy human subjects randomized to standard diet or AGE-restricted diet for 4 months identified significant, positive changes in insulin, leptin, 8-isoprostane and NF-KB levels in patients with diabetes but reductions in only serum CML and 8 isoprostanes in healthy subjects (Uribarri 2011). Based on animal and human data to date, it seems likely that dietary AGE may significantly impact AGE levels even in

healthy individuals despite somewhat low absorption. This is not a uniformly accepted finding. Controversy remains about the importance of dietary AGE.

Absorption and Effects of Ingestion of AGE

Several studies have documented impacts of short term AGE feeding on cellular processes. One of the earliest studies of AGE diet by Koschinsky et. al. evaluated the effect of a single meal containing egg white cooked with or without fructose (with=AGE; without=control) in 38 participants with type 2 diabetes. It was this study that first showed that not only did AGE increase with dietary consumption but that AGE levels remained elevated in persons with both diabetes and severe renal disease for more than 48 hours after ingestion (Koschinsky 1997). This provided impetus for research into whether AGEs could be responsible for some of the tissue damage which is present in diabetes.

A Greek group evaluating the effects of exogenous AGE on serum AGE in healthy women and women with polycystic ovarian syndrome (PCOS) studied the effects of a single high AGE meal on serum AGE in 36 women. The meal consisted of "overcooked" chicken nuggets, potato chips, meringue and Coke. They found increases in serum AGE in both normal women and women with PCOS (Diamanti-Kandarakis 2006). In this study a competitive AGE ELISA developed by the researchers was used to assess serum AGE content. An AGE profile of the test meal was not included. In addition, the researchers did not document negative effects of AGE, instead simply reporting that dietary AGE increased serum AGE.

A 2007 study attempted to show that dietary AGE had negative physiological consequences. Forty-four stable diabetic participants and 10 healthy participants were given an oral AGE challenge beverage containing 1.8×10^6 units of AGE but no carbohydrate or lipid (being based primarily on diet Coke). Brachial artery flow mediated dilation (FMD) was then

monitored for 72 hours. Maximal arterial dilation after ischemia decreased significantly with no changes in glucose or VCAM-1. This decrease in FMD is thought to be indicative of impaired endothelial function resulting from the AGE bolus (Uribarri 2007).

In a 2007 study by the same group, 20 participants with type 2 diabetes were given isocaloric diets with identical ingredients but which were prepared using different cooking methods in order to obtain high and low AGE test meals followed by measurement of endothelial function using FMD and Laser-Doppler flowometry. Each participant was fed the low AGE diet on day 4 of the study and the high AGE on day 6 followed by FMD monitoring during each phase. Again, FMD was found to decrease significantly more after the high AGE meal (a decrease which was1.5 times greater than the low AGE meal change). AGE did not affect glucose, triacylglycerol levels or insulin. No significant changes in inflammatory markers were found (Negrean 2007). The researchers did find a decrease in leptin after high AGE feeding. They also found decreases in adiponectin and increases in VCAM-1 and e-selectin. (Stirban 2008). Another study by the same group involving 13 persons with type 2 diabetes found that a high AGE test meal significantly impaired macrovascular FMD (Stirban 2006).

In a cross-over study of 11 patients with diabetes fed two weeks of high AGE followed by two weeks of low AGE diets with 1 one week washout between, serum AGES were found to increase by almost 65% on the high AGE diet and decreased by 30% on the low AGE diet. In a six week study of 13 patients with diabetes, C-reactive protein was found to increase on the high AGE diet and decrease on the low AGE diet with AGE LDL also decreasing on low AGE diet along with VCAM-1 (Vlassara 2002).

Others have disputed that AGEs are significantly absorbed or transported in the body. A 2007 review by Ames discusses a smaller body of the existing literature and reports that the data

concerning absorption is very spotty (Ames 2007). In one study of CML feeding involving rats, 26-29% of CML fed was recovered in the urine, 15-22% in the feces, 1.4% in kidney tissue, very small amounts in liver tissue, but half of the CML fed was not accounted for. Some have even suggested that CML is degraded by colonic microflora, but unpublished data investigating this was not very compelling (Ames 2007). Further complicating the picture, a study of hemodialysis patients found that serum CML was actually a positive predictor of survival (perhaps indicating good nutritional intake). (Schwedler 2002)

A review article suggests that genetic factors may be more important in determining AGEs in the serum than dietary intake. In non-diabetic twins, CML levels were found to be determined by genes not influencing blood glucose instead of diet (Ahmed 2005). In addition CML levels in serum have been found to correlate with retinopathy but not with nephropathy in diabetics (Ahmed 2005). Others maintain that CML and dietary intake are highly correlated. One study by Uribarri, Cai et. al found that consumption of dietary AGEs assessed by 3 day food record correlated with circulating CML (r=0.415). (Uribarri, Cai 2007) More recently Semba et. al. found no relationship between dietary records and circulating CML (Semba 2012).

Long-term AGE Ingestion

Very little has been studied in relation to the effects of long-term AGE ingestion on indicators of metabolic syndrome, kidney disease, and more. A very suggestive animal study was done by Cai et. al in 2008. In it 66 aging, male C67BL/6 mice were assigned to one of three diets: a 40% calorie deficient diet balanced in mineral and vitamins (CR diet), the same diet with the addition of AGE by extended heat exposure (CR-high diet) and an open formula (Reg) diet. Food consumption was monitored daily for four weeks and then after establishing daily intake, pair feeding began. Mice were followed for 112 weeks and blood was taken at 16 and 112 weeks of

age when sacrifice occurred for those mice who had not already expired. The micronutrient composition among the three diets was identical; the only difference was heating. The CR mice had a 45% lower consumption of protein and fat-associated dietary AGEs compared to the Reg mice due to the energy restriction. However, the CR-high mice consumed 2 times the AGE of the calorie restricted mice and 1.8 times greater than the Reg mice. After 56 weeks, the CR-high mice had higher body weight than the CR mice in spite of identical energy intake. Lifespan was also longer in the CR mice. Surprisingly lifespan was shorter in the CR-high mice than in those on the regular diet. (Cai 2008). This is a very surprising finding, one that points out the urgency of finding out what impact AGE could have in humans.

A few longer interventions in humans have been reported. In one study overweight and obese individuals were randomized to a crossover trial involving two weeks on a low and high AGE diet (Harcourt 2011). Only 11, male participants aged 18 to 50 were involved in this study. Fat was held constant between the two diets at 30% with AGE being the primary variable; however, AGE was calculated based on the Goldberg study. Foods consumed were similar but not identical. For example, the low-AGE lunch might include an apple and an avocado and ham sandwich without crusts with diet lemonade whereas a high-AGE lunch might include one apple and a toasted bacon sandwich with crusts and with a cola beverage. The primary outcome was renal function, and the researchers found that albumin to creatinine ratios were improved following the low AGE diet in obese individuals. Plasma AGE increased following high AGE consumption. Plasma monocyte chemoattractant protein-1 (MCP-1) was increased after consumption of the high AGE diet as was the plasma macrophage migration inhibitory factor (MIF). Other cytokines were no different including soluble RAGE (sRAGE), NF-κβ, Interleukin-6 (IL-6) and CRP (Harcourt 2011). Some drawbacks to this study design include likely

differences in type of fat in the study due to the dietary approach used. The distribution of calories from various types of fat (saturated, monounsaturated, polyunsaturated) was not described in this study; however, given that the low-AGE meal plan seems to be very low in fat yet diet composition was retained at 30% fat, it appears that the addition of olive oil and avocado, both mono-unsaturated fats, to some meals, was used to maintain this percentage. This may confound inflammatory data given that saturated fats are thought to be inflammatory (Holzer 2011).

Vlassara et. al. performed a similar type of study involving two arms: one consisting of a two week crossover trial involving 11 diabetic subjects and another involving 13 diabetic subjects in a 6 week study (Vlassara 2002). In this study the diets had similar calorie, protein and fat percentages but differed 5 fold in CML content. The high AGE diets met National Cholesterol Education Program Step 1 diet guidelines and American Diabetes Association guidelines. Thus they were not high in saturated fat (<10%). Serum AGE was measured by ELISA for CML. In the crossover trial, subjects followed either a low or high AGE diet for two weeks, had a one week washout period, then consumed the high AGE diet for two weeks. Both diets were low in fat, differing only in AGE content. For the six week trial, the 13 subjects were randomized to either the low or high AGE diet for 6 weeks, creating very small study groups. However, CRP increased by 35% on high AGE diet and decreased by 20% on low AGE. VCAM-1 decreased by 20% on low AGE and increased by 4% on high AGE. Serum AGEs increased by 28% on high AGE and were reduced by 40% on low AGE. Unfortunately almost no data on the dietary interventions was included with this report, making it difficult to evaluate any confounding dietary factors which may have been present.

Mechanisms of Damage Caused by AGE

A key review study by Brownlee has defined ways in which AGEs may damage target cells leading to diabetic complications. Three primary means of damage were defined.

1) Modification of cellular proteins by glycation could modify their function as well.

2) Extracellular proteins or other molecules modified by AGE might interact differently with their receptors.

3) Receptor mediated AGE oxidative processes may also cause cellular damage. (Brownlee 2001).

Most mechanistic explanations of how AGE may cause oxidative damage suggest that AGEs exert influence via receptors. RAGE receptors may be expressed on the surface of a variety of cells including neurons, monocytes, endothelial cells, fibroblasts and more (Kalousova 2005). The RAGE receptor may then activate signal transduction pathways including extracellular signal related kinase (ERK kinase), mitogen activated protein kinases (p38 ^{MAPK}), c jun terminal kinases (JNK kinases) and nuclear factor kappa beta (NF-κβ). Thus, transcription of genes may be stimulated which will up-regulate production of growth factors such as TNF-α, IL-1 and IGF-1 and adhesion molecules such as ICAM-1 and VCAM-1. In addition, it is suggested that DNA may also undergo glycation. Thus, AGE modification of DNA could affect regulatory and epigenetic processes at the DNA level (Ramasamy 2005).

Alternatively, in individuals some RAGE may be differentially spliced, forming soluble RAGE (sRAGE) which may act as a decoy receptor aiding in secretion of AGE (Barlovic 2011). sRAGE may actually consist of two isoforms: "the extracellular domain of wild type full-length RAGE, which results from proteolytic cleavage at the cell surface" and "an endogenous secreted isoform lacking a transmembrane domain" (Colhoun, 2011): sRAGE and esRAGE, respectively.

In addition, it has been discovered that the RAGE receptor has multiple ligands and that during an inflammatory response, the binding of the ligand to RAGE actually leads to increased expression of RAGE (Fritz 2011). This is one means by which a short-term inflammatory response could become a chronic state. The ligands for RAGE identified so far include AGEs, S100 proteins (a protein family consisting of 25 members which have metal ion binding properties), amyloid beta and amyloid fibrils, high mobility group box 1 protein (HMGB1) and beta2-integrin macrophage-1 antigen (Fritz, 2011). Thus, questions remain regarding whether all ligands for RAGE have been identified, which are most important, and how they may interact or compete with the RAGE receptor.

Other mechanisms of AGE damage have also been proposed. LDL modification by endogenous AGEs may be a contributing cause to the cardiovascular disease often seen in subjects with diabetes; however dietary AGEs may also contribute. One study of 24 participants with diabetes who followed a high AGE or low AGE diet for 6 weeks found that LDL from the patients on the high AGE diet was more glycated. In addition, when this glycated LDL was added to human umbilical vein (HUVEC) cells, ERK 1/2 was phosphorylated in a time and dose dependent manner compared to low AGE LDL or native LDL. Likewise, NF-κB was also stimulated by high AGE LDL. (Cai 2004)

In both cellular models and in Sprague-Dawley rats and non-obese diabetic mice subjected to AGE treatment or ingestion, AGEs were found to be toxic to the beta cells of the pancreas. Beta cells exposed to AGEs developed glucose stimulated insulin secretory defects and loss of manganese superoxide dismutase (MnSOD) activity. Rats exposed to AGEs developed progressive insulin secretory defect, generation of superoxide, and beta cell death (Coughlan

2011). Which mechanisms of AGE damage will be determined to be most important may depend somewhat on the age and health of the population in question.

INTERRELATIONSHIPS: BMI, AGE, sRAGE, AND DISEASE

It has become fairly well established that AGE may contribute to oxidative stress in renal disease and diabetes and that endogenous AGE production is increased in diabetes and excretion inhibited in chronic kidney disease (Ramasamy 2005; Semba 2009; Brownlee 2001). In addition, literature suggests that AGEs are involved in inflammatory processes (Ramasamy 2005; Brownlee 2001). The extent to which elevated circulating AGEs are the cause or symptom of disease and how AGEs, RAGE and sRAGE may be influenced by factors such as body fatness and lifestyle is less clear.

In several studies, fatness and sRAGE do appear to be linked. Koyama et. al. reported an inverse correlation of components of the metabolic syndrome with sRAGE. Components demonstrating this inverse relationship included body mass index, TG, HbA1c and insulin resistance with BMI in both diabetic and non-diabetic participants. This study involved 203 participants, but notably, the population was homogenous (Japanese), older (~56 years of age), and with a narrow BMI range (BMI 23.2 ± 3.3) (Koyama 2005). Another Japanese group also found sRAGE to be inversely correlated with BMI, waist circumferences, AGE intake and alcohol intake (Yamagishi 2006). This study involved 184 non-diabetic subjects. This group was again homogenous (Japanese), older (66.7 ± 9.1) and with a narrow BMI range (23.2 ± 3.7). CML was used as a proxy for AGEs in this study.

Norata et. al. also reported that sRAGE was inversely correlated with BMI, waist/hip circumference and fasting glucose with a positive correlation in apolipoprotein A-1. This study

included 176 healthy subjects. Again participants were homogenous (Italian), older (mean age of 62.4 ± 11.6 years), but with a somewhat wider BMI range (BMI 27.4 ± 3.6). (Norata 2009)

A third study by Semba et. al. used DEXA to establish body fatness and found that total fat mass, truncal fat mass and appendicular fat mass were each inversely associated with serum CML when age, sex, BMI, blood pressure, TG, HDL and renal function were controlled for. This suggests that body fat affects CML and possibly other AGEs perhaps because AGEs may be stored in fatty tissue (Semba 2011). This study involved 592 men and women who were older (age 55-75), 62% Caucasian with a BMI range of 23-31. Thus, the link between sRAGE and fatness seems to be established based on several studies done in separate populations. This suggests one possible strategy to improve serum sRAGE concentrations might be the perpetual lifestyle modification recommendation: weight loss.

It has also been noted in a recent review that two studies have found statins to raise sRAGE levels and another statin trial has found no effect (Ramasamy 2011). sRAGE levels have also been linked to white race, and higher adiponectin levels, but dietary influences on sRAGE have not been evaluated (Ramasamy 2011).

A study by Diamanti-Kandarakis et. al. found a correlation between AGEs and BMI. This study involved 60 women aged ~20-32 with BMI 19-24 (Diamanti-Kandarakis 2009). The study described here earlier by Yamagishi et. al. also described a link between AGEs and BMI. Given more limited data in this area, the link between AGEs and fatness is suggestive.

Finally the study by Yamagishi et. al. also identified a correlation between sRAGE and AGE. However, they found a positive correlation between the two. This seems counter-intuitive given research suggesting the AGEs are increased with increasing BMI whereas the link is opposite for sRAGE. This group of researchers hypothesized that sRAGE may reflect tissue

RAGE expression and that sRAGE may increase along with AGE in order to mount a counter-defense (Yamagishi 2006).

A separate Japanese study found a relationship between AGE and esRAGE in 107 patients with type 2 diabetes. Serum AGE and sRAGE were both highest among participants with diabetes who were also on hemodialysis. Serum esRAGE was correlated with BMI, duration of diabetes, serum creatinine, high density lipoprotein and CML and pentosidine in this study (Gohda 2008).

However, in a study of older, Caucasian, Swedish men, CML concentrations were not related to sRAGE nor to energy, carbohydrate, total fat, type of fat, or vitamin intake. In this study BMI and endothelial cell activation were found to be linked, but sRAGE and endothelial activation were not. The relationship between BMI and sRAGE or CML was not reported in this study (Sjogren 2007). Thus, the relationship between AGE and sRAGE seems a bit tenuous for now. Further work needs to be done in this area.

In addition, consistency of sRAGE/esRAGE data is questionable. One head to head comparison of these two forms of RAGE: sRAGE and es RAGE has been done in a German population. In it 110 persons with type 2 diabetes were recruited and subjected to three 24 hour urine collections and fasting blood tests. In this study sRAGE and esRAGE were found to be correlated by only at r=0.57. esRAGE levels were five times lower than sRAGE values. Very little was decided in this study given that sRAGE and es RAGE were not shown to be associated with glucose control or macrovascular disease (Humpert 2007).

An animal model developed by Ueno and Koyama has sought to determine whether RAGE is involved in adiposity and to explore the relationship of RAGE with progression of atherosclerosis (Ueno 2010). C57BL/6J RAGE $^{+/+}$ and RAGE $^{-/-}$ mice were produced, crossed and

intercrossed to generate $ApoE^{-/-}RAGE^{+/+}$ and $ApoE^{-/-}RAGE^{-/-}$ mice. These mice were then randomly assigned to atherogenic diet (20% of kcals from coca butter) and standard chow diet from 6 weeks to 20 weeks of age. The $ApoE^{-/-}RAGE^{-/-}$ mice fed with either diet had decreased plaques even though no differences in cholesterol or glucose were observed. The $ApoE^{-/-}RAGE^{-/-}$ mice also gained significantly less body weight despite a lack of difference in food intake. Researchers also found that plaque area was positively associated with epididymal fat weight and negatively with serum adiponectin. Thus, it appears that mice who are deficient in RAGE have improved body fatness and less atherogenesis, an intriguing finding should it also be found to be true in humans.

Other relationships of AGE to metabolic indicators have also been described. In a study of 51 Greek women, of which 29 had PCOS, AGE was found to be higher in the women with PCOS. AGE was also found to be increased in women with increased testosterone levels. A positive correlation was also noted between AGE and free androgens, waist to hip ration, insulin, HOMA and RAGE. Negative correlations with AGE were observed for glucose/insulin ration and the quantitative insulin sensitivity check index (QUICKI). (Diamanti-Kandarakis 2005).

AGEs AND ADIPONECTIN

A less well studied area of AGEs research involves how AGEs and adiponectin relate. Adiponectin is an adipokine, or hormone released from adipose tissue, which is decreased in diabetic subjects and those with elevated BMI (Rosen 2006; Hotta 2000). In a study of 108 overweight patients, CML was found to inversely correlate with adiponectin levels. Furthermore, adiponectin seems to be protective against endothelial dysfunction induced by AGEs (DelTurco 2011). However, within the field of adiponectin research there is disagreement over which form

of adiponectin is the active form of hormone given that adiponectin circulates in multimeric forms (Rosen 2006).

Another human study in a Korean population found sRAGE to correlate with adiponectin, IL-6 and to be inversely correlated with BMI, waist circumference, blood pressure, triglyceride, fasting glucose level and insulin resistance. They also found lower sRAGE levels in participants with type 2 diabetes. (Choi 2009)

One group of investigators has tried to evaluate how AGEs may modify serum adiponectin concentrations. This group hypothesized that since oxidative stress has been known to decrease adiponectin levels in rats and since AGE-RAGE interaction may increase oxidative stress, AGE-RAGE interaction may induce adiponectin gene suppression (Maeda 2011). To study this effect, they used pigment epithelium-derived factor (PEDF), a glycoprotein which is known to block AGE-RAGE induced inflammatory reactions in cells, to treat differentiated adipocytes which had also been treated with AGE. They found that AGEs increased oxidative stress in the adipocytes but that this effect was blocked by PEDF. The AGEs up-regulated decreases in adiponectin mRNA, and the PEDF treatment completely ameliorated this effect.

This area of research is particularly intriguing as this may another way in which AGEs exert their detrimental physiological effects: via suppression of adiponectin. This also poses a possible therapy for persons with diabetes: lowering of AGEs within the diet in order to up-regulate adiponectin and suppress AGE-related cellular damage.

MACRONUTRIENT INTAKE, AGE, AND INFLAMMATORY MARKERS

Another area of research yet to be fully elucidated in the field of AGE/RAGE biology and nutrition includes the interaction between dietary macronutrient composition and serum AGE and sRAGE. Much has been made of the importance of low fat diet in lowering risk of cardiovascular

disease. However, cooking methods recommended for lowering fat intake such as broiling, grilling and roasting tend to increase concentrations of advanced glycation end products (AGEs) in foods. Current research suggests that AGEs increase oxidative and carbonyl stress, may decrease cardiovascular endothelial dysfunction, and contribute to cellular damage related to diabetes and chronic kidney disease. In addition increased body fat and body weight are associated with higher levels of AGE and decreased levels of an important receptor for AGE (sRAGE). AGE in the diet has also been associated with increased levels of CRP (Uribarri, Cai 2007).

Since low fat diet is often recommended for weight control in obese and overweight persons, it is important to determine to what extent a low fat diet using these commonly advised cooking methods may contribute to AGE load and whether such a low-fat diet may negatively affect inflammatory cytokines thought to be influenced by AGE. It is also unclear whether dietary AGE influences sRAGE. Does the body have some capacity to compensate for high AGE intake with higher sRAGE? In fact, given recent data suggesting there is no relationship between dietary AGE and serum AGE, it needs to be confirmed that dietary CML will result in increased serum CML. In the context of different types of diets which are isocaloric, will a low fat diet cooked using high heat methods have equivalent amounts of AGE compared to a high fat diet cooked in these ways? Will consumption of a low fat diet using these cooking methods have the same effects on serum AGE and sRAGE compared to a high fat diet?

CHAPTER III

METHODS FOR STUDY I AND II

These studies were approved by the Institutional Review Board Human Subjects Review Committee at Texas Woman's University (Appendices A and B).

STUDY I

Participants

Study I was part of a larger study. Briefly, 77 healthy adult participants between the ages of 20 and 45 were recruited from the Texas Woman's University campus community. Exclusion criteria included self-reported hypertension, hypercholesterolemia, diabetes, heart disease, kidney disease, cancer or metabolic disease. Smokers and those taking any medication which could affect blood lipids or glucose levels were also excluded. Frozen fasting sera were used for analysis.

Anthropometric and Biochemical Measurements

Potential participants reported to the research location on a Saturday morning following a twelve hour overnight fast. After obtaining informed consent, participants were screened. Anthropometric measurements (height, weight, waist circumference) were done by the same personnel.

Weight was measured to the nearest 0.1 pound using a beam scale, height to the nearest 0.25 inch using a stadiometer, and waist circumference to the nearest 0.25 inch at the level of the hip

bone using two consecutive measures with a non-elastic tape. Measurements were converted to metric units for reporting purposes.

A phlebotomist obtained fasting blood, which was then allowed to clot for 30 minutes and centrifuged at 3600 rpm for 15 minutes. Sera were separated, aliquoted and stored at -80^0 C until assayed.

Blood Sample Analysis

Sera were analyzed for triacylglycerol (TG), total cholesterol (TC), high density lipoprotein (HDL), and glucose using commercially available kits (StanBio, Boerne, TX). LDL was calculated using the Friedewald Equation ([LDL-chol] = [Total chol] - [HDL-chol] - ([TG]/5)). Adiponectin, insulin and HMW adiponectin were measured using enzyme linked immunoassay kits (ELISA) (RayBiotech, Norcross, GA; Alpco, Salem, NH; Alpco, Salem, NH). CML and sRAGE were also measured using ELISA (Microcoat, Bernried, Germany; Biovendor, Karasek, Czech Republic). See Appendix C for the methods used for these analyses.

Statistical Analyses

Potential numerical outliers were identified using box plots and 1.5 times the Q_1 to Q_3 interquartile range. Data were analyzed with and without outlier data for comparison. Pearson's correlations were performed for all sera and anthropometric parameters. SPSS version 19.0 for Windows was used to perform statistical analyses including descriptive statistics, analysis of data for normality, and correlations.

Based on the techniques to identify outliers outlined above, the data from five (5) participants were excluded. Reasons for exclusion from the study group included having extreme

values for sRAGE (n=2), CML (n=2), or insulin (n=1). Statistically significant relationships were present when outlier data was included; however, the strength of the correlations were slightly increased when outlier data was excluded.

STUDY II

Participants and Recruiting

Male and female adult participants ages 20-45, BMI 27-35 (overweight or class 1 obesity) without self-reported hypertension, heart disease, diabetes, kidney disease, metabolic disease or cancer were recruited from the TWU campus community and the larger Denton community. Methods of recruitment included use of course advertisements through the course management system for the University, Portal announcements geared toward TWU faculty, staff and students, and print announcements on university bulletin boards. In addition, local doctor's offices, primarily family practice physicians, were contacted and asked to share the flyer with eligible, interested patients. Local gyms, clubs and community organizations were also contacted and asked to share information about the study with their members. A Facebook page was created in which updates and announcements were provided. Instructors at the University of North Texas were also contacted and asked to share information about the study with their students. See Appendix D for copies of the recruitment flyer and Portal/Facebook flyer.

Interested potential participants contacted the primary researcher by phone or e-mail. If the first contact was by phone, the researcher requested the potential participant's e-mail address in order to send information on study criteria and how to determine study eligibility. Interested potential participants contacted the researcher again to set a time to meet with the participant on campus, explain and sign the consent form, and complete the screening process. Following the

completion of informed consent, participants were screened to see if they met study criteria, including being free of hypertension, diabetes, cardiovascular disease, kidney disease, cancer, or metabolic disorders. Only participants without significant medical histories were allowed to participate. Smokers were excluded from the study, as were those taking anti-inflammatory medications, lipid lowering drugs, or those who were pregnant or lactating. After screening was complete, participants were assigned a time to come to campus for the study. See Appendix E for a sample of the scripts for contact with the researcher, the study flyer, and the electronic version of the study flyer. See Appendix F to review the form used for screening participants.

Nutrition Instrument

Participants were instructed to fill out a five-day food record based on the five days immediately preceding the study. A food journal form was used for this purpose. (See Appendix G for an example of the food journal.)

Anthropometric, Biochemical Measurements and Study Protocol

All participants were scheduled to consume both study diets. Diet A was a low-fat, high AGE diet (LFHA). Diet B was a high fat, high AGE diet (HFHA). Each diet was consumed for one day with a series of blood draws following the first test meal and a follow-up fasting blood draw the day after consumption of the test diet. A two-week washout period was inserted between each test day's diet. Please see additional information on the diet in the next section "Diet Intervention".

On day one of the study, participants reported to the lab for anthropometric measurements. A portable stadiometer was used to measure height to the nearest one-quarter inch, and a beam scale was used to measure weight to the nearest 0.5 pound. Height and weight measurements were converted to centimeters and kilograms for reporting, and body mass index

(BMI) was calculated in kg/m^2. Waist circumference was measured with a non-stretch tape measure to the nearest 0.5 cm at the narrowest part between the lower costal (10^{th}) rib border and iliac crest. Finally, percentage body fat was measured using bioelectrical impedance analysis (BIA) using a Tanita electronic scale. See Appendix H for a copy of the data collection form.

Five blood draws were conducted during each phase of the study. The first blood draw was done at baseline during the first trip to the lab with follow-up blood draws to include 1 hour, 2 hour and 3 hour post-prandial draws after consumption of the first test breakfast meal. After completion of the 3 hour post-prandial blood draw, participants consumed lunch. They consumed an afternoon snack, dinner and a bedtime final snack over the course of the remainder of the day and returned to the lab the next morning for another fasting draw.

After a two week wash-out period, participants returned to campus for the second phase of the study. Anthropometric measurements were done immediately prior to the second dietary intervention period by the same personnel. The same protocol was used for both dietary interventions. Each blood draw was be performed by a trained nurse or certified phlebotomist.

Diet Intervention

Participants received two isocaloric study diets: one containing 20% of calories from fat, the other with 41% of calories from fat. The fatty acid distribution of both diets was 1:2:1 (MUFA:PUFA:saturated fat). High AGE cooking methods were used for each: broiling, roasting, baking, and frying. See Appendix I for a copy of the menus.

Study foods were prepared in the Food Preparation Laboratory or Sensory Laboratory and were provided for breakfast, lunch and dinner daily during the two one-day study periods. Food was consumed in the lab or boxed and given to the participant to be consumed at home

according to the need of the participant. Dietary macronutrient content was calculated initially using Nutritionist Pro, then confirmed by proximate analysis by an independent laboratory: Pope Labs. See Appendix J for a copy of the analyses. AGE content in study foods was calculated initially using the tables of Uribarri (2010). It was later measured using ELISA and was compared using several methods to prepare foods for AGE ELISA analysis. See Appendix K for methods to analyze AGE in the study foods. Participants were allowed to eat only the study foods during each day-long intervention period.

Biochemical Analysis

Sera were analyzed for glucose, triacylglycerol (TG), total cholesterol (TC), and high density lipoprotein (HDL) using commercially available kits (StanBio, Boerne, TX). LDL was calculated using the Friedewald Equation ([LDL-chol] = [Total chol] - [HDL-chol] - ([TG]/5)). HMW adiponectin was measured using enzyme linked immunoassay kits (ELISA) (Alpco, Salem, NH). CML, CRP, and sRAGE were also measured using ELISA (Microcoat, Bernried, Germany; Alpco, Salem, NH; Biovendor, Karasek, Czech Republic). See Appendix B for the methods of these analyses, Appendix L for CRP.

CHAPTER IV:

SOLUBLE RECEPTOR FOR ADVANCED GLYCATION ENDPRODUCTS (SRAGE) CORRELATES INVERSELY WITH INDICATORS OF ADIPOSITY

A Paper to Be Submitted for Publication in *Acta Diabetologica*

Kathleen E. Davis

ABSTRACT

Obesity is a public health crisis because of its high incidence and its link to diabetes and cardiovascular disease, conditions associated with an inflammatory state. Advanced glycation endproducts (AGEs) may contribute to inflammation within the body by interacting with the receptor for AGEs (known as RAGE), but the soluble receptor for this compound may reduce serum concentrations, thereby decreasing oxidative stress. The purpose of our study was to explore the relationship among measures of adiposity, AGEs, and their soluble receptor in a diverse group of healthy young adults. Anthropometrics were obtained from 72 participants, and sera were analyzed for n-(epsilon)-(carboxymethyl)-lysine (CML), a measure of advanced glycation endproducts and for sRAGE (n=72). Total adiponectin, high molecular weight (HMW) adiponectin, insulin, total cholesterol, high density lipoprotein, and triacylglycerol were also assessed. sRAGE was inversely correlated with weight (r= -0.364; p=0.002), waist circumference (r= -.334; p=0.004), and body mass index (BMI) (r= -0.364; p=0.002). This is the first time such associations have been found in a group of ethnically-diverse, healthy, young adults. HMW adiponectin also correlated with sRAGE (r=0.416; p=0.02). Controlling body weight and

improving insulin resistance, factors tied to adiponectin concentrations, may reduce inflammation via up-regulation of sRAGE.

INTRODUCTION

During the past several decades, obesity has become a public health crisis [1-2], and the incidence of obesity has risen at an alarming rate in both industrialized and developing countries. Currently, 67% of Americans are either overweight or obese. Obesity has been clearly linked to diabetes and cardiovascular disease and as such is an important public health issue [3]. As obesity and sedentary lifestyle have increased, so have the prevalence of other inflammation-related conditions such as type 2 diabetes and metabolic syndrome, which are characterized by abdominal obesity, hyperlipidemia, hypertension and insulin resistance [2].

Advanced glycation endproducts are a family of compounds formed when reducing sugars react with amino acids or other substances, forming glycosylated molecules. They may contribute to oxidative stress within the body by interacting with the receptors for advanced glycation endproducts (RAGE), the immunoglobulin super family of receptors [4]. RAGE has a single trans-membrane domain followed by a cytosolic tail with the N-terminus of the V domain acting as the ligand binding site, and the cytosolic tail functioning in RAGE-induced intracellular signaling [5-6]. Truncated forms of RAGE have also been reported which may result from alternative mRNA splicing or proteolytic cleavage from the cell surface [5]. The variant which lacks both the cytosolic and the trans-membrane domains is called the soluble receptor for advanced glycation end products (sRAGE) [5-7]. Because sRAGE retains its ability to bind circulating ligands, it may play an important role in attenuating the adverse effects associated with RAGE signaling [5].

RAGE is widely expressed in a variety of tissues (heart, lung, skeletal muscle, and vessel wall) and cell types (monocytes/macrophages and lymphocytes) [4]. The activation of RAGE by its ligands (including AGEs) results in induction of oxidative stress and expression of pro-inflammatory cytokines and chemokines. Therefore, serum sRAGE has the potential to diminish AGE-RAGE interaction by binding circulating AGEs, resulting in decreased inflammation [6, 8]. In addition, AGE-RAGE signaling may be decreased via up-regulation of circulating sRAGE [5-6].

Obesity is a public health crisis because of its high incidence and its link to diabetes and cardiovascular disease. Obesity is characterized by a chronic, systemic state of low-grade inflammation. Thus, it is logical to assume that sRAGE signaling may be reduced in obesity and even in overweight conditions. Studies done in older, homogenous populations of adults and children suggest that sRAGE is depressed in obese individuals compared to normal weight individuals [9-16]. In addition, sRAGE is currently being evaluated for its utility in identifying cardiometabolic risk in populations from adults to children [13, 17-19]. However, the relationship of sRAGE with factors which may predict its levels have only been partially uncovered. In several studies sRAGE has been inversely correlated with obesity. However, none of these studies has been done in a diverse population. Moreover, there is conflicting data in the literature regarding sRAGE and abdominal adiposity in children and older adults [9-16]. Therefore, this study investigated the relationship among n-(epsilon)-(carboxymethyl)-lysine protein, a measure of advanced glycation endproducts, sRAGE, and indicators of adiposity in a group of young, healthy, ethnically diverse adults.

METHODS AND MATERIALS

This study was approved by the Texas Woman's University Institutional Review Board's Human Subjects Committee. Seventy-seven healthy adult participants between the ages of 18 and 45 were recruited from the university community. Exclusion criteria included self-reported hypertension, hypercholesterolemia, diabetes, heart disease, kidney disease, cancer or metabolic disease. Smokers and those on lipid- or glucose-lowering medications were also excluded.

Participants reported to the research location following a twelve hour fast. After obtaining informed consent, participants were screened for inclusion and exclusion criteria. Anthropometrics (height, weight, and waist circumference) were obtained for subjects selected for the study, and fasting blood was collected, allowed to clot for 30 minutes, centrifuged at 3600 rpm for 15 minutes, and stored at -80^0 C.

Sera were analyzed for triacylglycerol (TG), total cholesterol (TC), and high density lipoprotein (HDL) using commercially available kits (StanBio, Boerne, TX). LDL was calculated using the Friedewald Equation ([LDL-chol] = [Total chol] - [HDL-chol] - ([TG]/5)). Total adiponectin, insulin, high molecular weight (HMW) adiponectin, CML and sRAGE were measured using enzyme-linked immunoassay (ELISA) kits (RayBiotech, Norcross, GA; Alpco, Salem, NH; Alpco, Salem, NH; Microcoat, Bernried, Germany; BioVendor, Karasek, Czech Republic, respectively).

Potential numerical outliers were identified using box plots and 1.5 times the Q_1 to Q_3 interquartile range. Data were analyzed with and without outlier data for comparison. Pearson's correlations were performed for all sera and anthropometric parameters. SPSS version 19.0 was used to perform statistical analyses including descriptive statistics, analysis of data for normality,

and correlations. ANOVA was used to compare differences in parameters based on BMI classification and waist circumference.

RESULTS

Of 77 participants from whom data were obtained, 72 were included in the study. Five subjects were excluded due to having extreme values for sRAGE (n=2), CML (n=2), or insulin (n=1). The study population included 22 male subjects (Age: 26.4 ± 4.5 years; Ethnicity: Asian 41%, Caucasian 36%, Hispanic 18%, African-American 5%; Body Mass Index (BMI): 23.9 ± 2.0; Overweight and Obese: n=6) and 50 female subjects (Age: 23.6 ± 5.2 years; Ethnicity: Asian 18%, Caucasian 52%, Hispanic 14%, African-American 14%, Other 2%; BMI: 24.3 ± 4.2; Overweight and Obese: n=18). A greater proportion of females in the study were overweight (36% compared to 27% for males). While the BMI differences between the sexes were not statistically significant, the groups were significantly different in age (p=0.04).

While participants' biochemical data was noted to be different between males and females for HDL (Males: 1.04 mmol/L ± 0.28, Females: 1.35 ± 0.31, p=0.007), TG (Males: 1.11 mmol/L ± 0.54; Females: 0.64 ± 0.44, p=0.01), and HMW adiponectin (Males: 1.9 µg/ml ± 1.2, Females: 3.2 ± 1.8, p=0.03), there was no difference with regard to TC (Males: 4.43 mmol/L ± 0.83, Females: 4.09 ± 0.78), adiponectin (Males: 0.54 µmol/L ± 0.57; Females 0.52 ± 0.37), insulin (Males: 34.7 pmol/L ± 13.9; Females: 43.8 ± 31.3), CML (Males: 722 ng/mL ± 198, Females: 844 ± 323) or sRAGE (Males: 552 pg/ml ± 223, Females: 626 ± 256).

Factors inversely correlated to sRAGE included weight (R= -0.364; p=0.002), waist circumference (R= -0.334; p=0.004), and BMI (R= -0.364; p=0.002). As is shown in Table 1, HMW adiponectin was positively correlated with sRAGE concentrations (0.416; p=0.02). When

ANOVA was used to compare BMI classification with sRAGE, sRAGE was significantly different only when comparing normal weight to obese participants, but there is a non-significant decrease in sRAGE as BMI increases (Figure 1). In addition, sRAGE was found to be significantly different between participants with a high waist circumference (>102 cm in men; >88 cm in women) indicating increased health risk compared to those with normal waist circumference ($p=0.03$).

Using linear regression model, HMW adiponectin, TC, BMI and CML were found to be significant predictors of sRAGE. However, when combined, these predictors explained only 47% of the variance in sRAGE as other factors such as dietary intake of AGEs, undetermined genetic factors or underlying health status may also influence sRAGE.

DISCUSSION

In the present study, we have reported for the first time a strong, inverse relationship between measures of adiposity and circulating sRAGE in young adults of diverse ethnicity between ages 18 and 40. Our observations are similar to those reported in four earlier studies in homogenous older Japanese and Italian adults of mean age 45 to 66 years [9-12] and in children [13]. In contrast to these studies, our study did not find an inverse correlation between sRAGE and glucose, insulin, or TG in young, healthy ethnically-diverse adults.

Our study points to a number of similarities and differences compared to results reported by others [10-16]. Unlike Choi [14] we found a significant correlation between sRAGE and HMW adiponectin, but not total adiponectin. Whereas Del Turco *et. al.* found an inverse correlation between CML and adiponectin [15], this finding was not observed in our data. However, similar to Yamagishi [10], we found a positive correlation between CML and sRAGE.

(R=0.237, p=0.05). Also, we did not find an inverse relationship between CML and adiposity as did Semba *et. al*. [16].

sRAGE appears to be an anti-inflammatory marker, correlating inversely with body weight and positively with HMW adiponectin. However, it also correlates positively with CML, which is thought to be a pro-inflammatory surrogate marker of AGEs. One explanation for this anomalous observation may lie in possible induction of sRAGE by increasing CML load.

Because oxidative stress decreases adiponectin expression, Maeda et. al. hypothesized that AGE-RAGE interaction may further exacerbate oxidative stress by suppressing the adiponectin gene [20]. These investigators reported that AGEs increased oxidative stress in rat adipocytes and that AGEs down-regulated adiponectin mRNA. This area of research is particularly intriguing as this may be another way in which AGEs exert their detrimental physiological effects: via suppression of adiponectin. This may be a potential therapy for persons with diabetes: lowering of AGEs within the diet in order to up-regulate adiponectin and suppress AGE-related cellular damage.

One limitation of our preliminary study is that we used CML as an indicator of AGE although there are several indicators of AGE. CML measurements have been found to correlate well with other markers of AGE [21-23].

While our observation of the relationship between sRAGE and adiposity in a young population is similar to earlier studies with older subjects, the relationships observed among other markers related to adiposity (glucose, insulin, adiponectin, Chol, TG) are dissimilar in many cases. The underlying reasons for these apparent discrepancies may lie in the differences in the study population with respect to ethnicity, age, and underlying health status.

ACKNOWLEDGEMENTS

This research was supported by Human Nutrition Research Fund of the State of Texas.

CONFLICT OF INTEREST

None.

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Table 4.1. Pearson's Correlations: Biochemical Parameters and Indicators of Adiposity

	Weight	**Waist**	**BMI**	**sRAGE**	**HMW Adiponectin**
Waist: r **Sig** **N**	0.830 0.000** 72				
BMI: r **Sig** **N**	0.806 0.000** 72	0.790 0.000** 72			
sRAGE: r **Sig** **N**	-0.364 0.002** 72	-0.334 0.004** 72	-0.364 0.002** 72		
HMWAdipo: r **Sig** **N**	-0.518 0.002** 34	-0.541 0.001** 34	-0.479 0.005** 34	0.416 0.016* 34	
CML:r **Sig** **N**	-0.169 0.164 69	-0.169 0.164 69	-0.038 0.754 69	0.237 0.050* 69	-0.175 0.337 32

*$p \leq 0.05$;

**$p < 0.01$

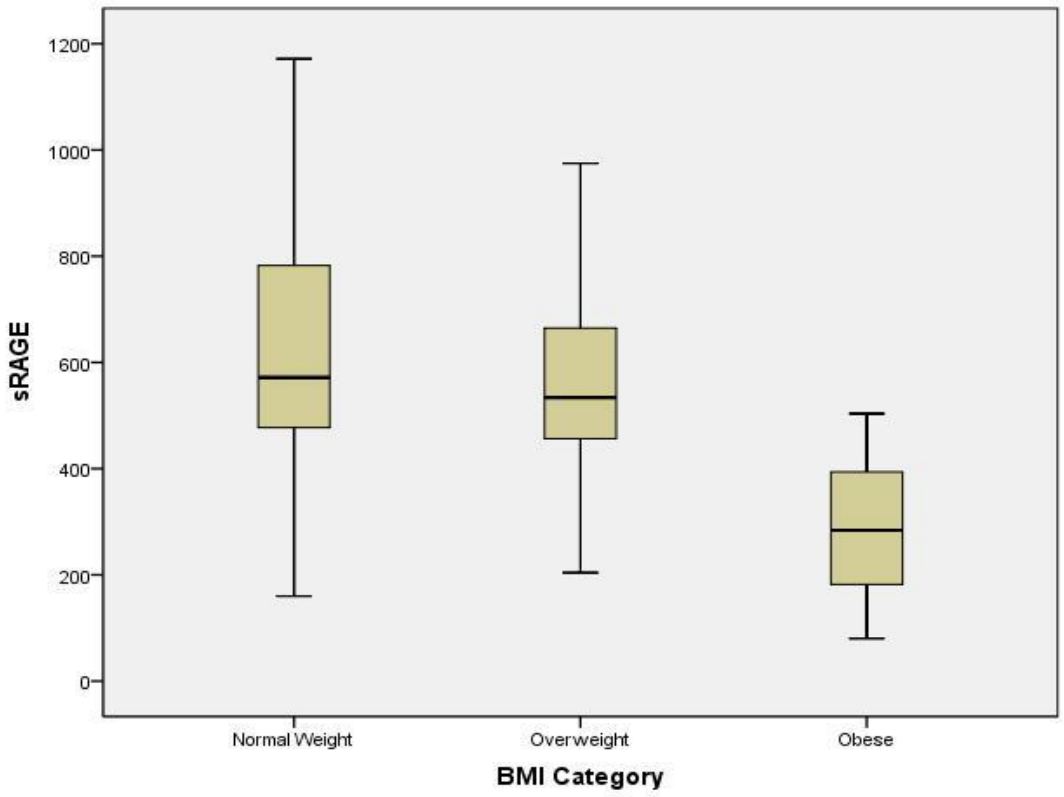

Figure 4.1. Relationship Between BMI Classification (Normal; Overweight; Obese) and sRAGE (expressed in pcg/ml). *

*Data expressed as means +/- SD.

CHAPTER V

ADVANCED GLYCATION END PRODUCTS, INFLAMMATION, AND CHRONIC METABOLIC DISEASES: LINKS IN A CHAIN?

A Paper Accepted for Publication in *Critical Reviews Food and Nutrition Science*

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ABSTRACT

Advanced glycation end products (AGEs) are a diverse group of compounds produced when reducing sugars react with proteins or other compounds to form glycosylated molecules. AGEs may form endogenously, and glycation of molecules may negatively affect their function. AGEs may also be consumed in food form with dietary AGEs reported to be particularly high in foods treated with high heat: baked, broiled, grilled, and fried foods. Whether dietary AGEs are absorbed in significant quantities and whether they are harmful if absorbed is a question under current debate. The American Diabetes Association makes no recommendation regarding avoidance of these foods, but many researchers are concerned that they may be pro-inflammatory and way worsen cardiac function, kidney function, diabetes and its complications and may even contribute to obesity.

INTRODUCTION

Advanced glycation end products (AGEs) are a diverse group of compounds formed when glucose or other reducing sugars react with amine groups linked to proteins, nucleotide bases or fatty acids, forming glycosylated products. Some of these products include carboyxmethyllysine (CML), hydroimidazolone, pentosidine, glucosepane, and others (Semba, Nicklett 2010).

The Maillard browning reaction in foods, a necessary precursor to the formation of AGEs, was first described by Louis Camille Maillard in 1912, and for many, the Maillard reaction is most familiar as something belonging to the realm of food science, affecting the color, flavor, and acceptability of cooked food. However, increasingly this reaction and the reactions which may follow it are being investigated for their role in negative physiological effects in the body.

The first indirect support for health implications of non-enzymatically glycated proteins came from observations of increased formation of hemoglobin A_{1c} in parallel with poor glucose control in persons with diabetes (Trivelli 1971). Hemoglobin A_{1c} is an Amadori product, a ketoamine formed as the ultimate product of a reaction between glucose and the free amine group on the valine residue of the hemoglobin beta chain (Saudek 2006). It is not an AGE, but it is the standard for determining adequacy of blood glucose control in diabetes today (Saudek 2006). It is used as an indicator of the 3-4 month average of blood glucose values, with goals for patients with diabetes set at a hemoglobinA_{1c} of less than 7% or 6.5% (Saudek 2006). While hemoglobin A_{1c} is not an AGE, this research became an important springboard for future AGE research. The implications of AGEs as possible pro-inflammatory compounds gained further steam and legitimacy when the receptor for AGEs (RAGE) was cloned in 1992 (Schmidt 1992; Semba, Nicklett 2010). Other receptors for AGE have since been identified.

WHAT ARE AGEs?

The Maillard browning reaction, which yields a yellowish-brown colored product, precedes the formation of AGEs. In this process, a reducing sugar and amine group react, non-enzymatically forming a Schiff base, which may then form more stable Amadori products (Wu 2011). The first part of this reaction is reversible, so AGEs may *not* result from the initial

browning reaction; however, the Amadori products, once formed, lead inexorably to the formation of AGEs. AGEs are produced when the Amadori products are further modified by dehydration, oxidation, rearrangement or other reactions, leading to the formation of cross linked or non-cross linked derivatives of sugar and other molecules (Barlovic 2011; Wu 2011). See Figure 1 (Sell 2012) for a detailed look at how AGEs are formed.

Although AGEs include pyrraline, pentosidine, carboxymethyllysine, glucosepane and some 16 plus other structures (Ames 2007), hemoglobin A_1c, though an Amadori product, is not an AGE. AGEs are found both in food and within the body. Among cross-linked AGEs are crossline, 2-(2-furoyl)-4(5)-(2-furanyl)-1H imidazole (FFI), glyoxal-lysine dimer (GOLD), methyl-glyoxal-lysine dimer (MOLD) and others (Wu 2011). Non cross-linked AGEs include CML, carboxyethyllysine (CEL), pyrraline, and argpyrimidine (Wu 2011).

The substances formed when reducing sugars interact with substances containing a free amino acid group may be called pre-AGEs. In addition, sugars in the body or in food may be oxidized to form dicarbonyls such as glyoxal (GO) and methylglyoxal (MG). GO and MG are not themselves AGEs and may have other fates, but are thought to have strong AGE potential and the ability to form cross-linked AGEs via reaction with arginine residues. The product of this reaction is the AGE hydroimidazolone. In addition, GO and MG may impact formation of reactive oxygen species. (Ames 2007; Wu 2012)

MEASURING AGEs

Although several procedures are available for measuring AGEs, neither a universally accepted technique nor normative values have been established. Immunochemical and instrumental methods are most often employed to measure AGEs. Immunochemical methods

(enzyme linked immunosorbent assay or ELISA) have the advantage of requiring less expensive equipment in order to begin quantification, but studies employing these methods have been criticized for being non-specific due to the nature of the antibodies employed and also for reporting AGE in "Units" per average portion (Goldberg 2004; Uribarri 2010; Ames 2008). Instrumental methods used to quantify AGEs in food have included liquid chromatography combined with fluorescence detection or mass spectrometry (HPLC, UPLC) or in some cases gas spectrophotometry (GC) (Ames 2008; Birlouez-Aragon 2004; Hull 2012; Assar 2009; Delgado-Andrade 2006, 2007; Drusch 1998; Yaacoub 2008). Instrumental methods are usually accurate, repeatable and express AGEs in milligrams (mg) per kilogram (kg) of protein, mg/100 grams (g) of food or mg/average portion size.

The problem in comparing published AGEs data are multiple. These include method used for quantification (instrumental versus ELISA) and antibody-dependent differences in ELISA assays that may measure different AGEs products. In addition, despite the identification of at least 20 AGE structures, CML has been most often reported in the literature as a measure of AGEs (Semba, Nicklett 2010). The use of CML as a proxy for measuring AGEs is based on early studies indicating that CML levels correlate directly with levels of other protein or lipid AGEs (Cai 2008; Brownlee 2001; Requena 1997). However, much remains to be understood about the physiologic significance of the large AGEs family of structures and which should be measured as a marker of AGEs load.

AGEs have multiple receptors including RAGE, AGERs 1, 3, and CD35 receptors, which will be discussed further in the section on "Metabolism and Pathophysiology". In addition, given the discovery that RAGE has multiple ligands and that during an inflammatory response, the binding of the ligand to RAGE leads to increased expression of RAGE (Fritz 2011), it is

reasonable to ask whether each ligand has an equal affinity for RAGE and whether CML is the most important ligand in promoting inflammation. The ligands for RAGE identified so far include AGEs, S100 proteins (a protein family consisting of 25 members, which have metal ion binding properties), amyloid beta and amyloid fibrils, high mobility group box 1 protein (HMGB1) and beta2-integrin macrophage-1 antigen (Fritz, 2011). RAGE is classified as a "pattern recognition receptor" because of its ability to allow binding by a diverse group of ligands (Ramasamy 2010). Questions remain regarding whether all ligands for RAGE have been identified, which are most important, and how they may interact with or compete for the RAGE receptor.

Some progress is being made in this regard. A recent study attempted to use UPLC with mass spectrometry (UPLC-MS) to analyze the glycating activity of AGE-precursors. This study identified five AGE structures which did not match any known product, but it also confirmed that CML and CEL were abundant AGEs formed from well-known AGE precursors such as MG GO and glucosone (Mittelmaier 2011).

Another problem in the AGE literature is that measured AGEs in foods vary based on the quantification method used. Roast chicken breast in which AGEs were quantified using ELISA is reported to have 5,245 kU of AGE per 90 g serving (Goldberg 2004; Uribarri 2010). Using UPLC-MS, a 90 g serving is reported to have 0.4 mg/ serving (Hull 2012). This compares to 825 kU of AGE per 90 g serving of canned salmon (ELISA) and 6.2 mg/90 g (UPLC) (Hull 2012; Goldberg 2004). Thus, according to ELISA measurements, broiled chicken is high in CML whereas canned salmon is low (Uribarri 2010). Conversely, according to UPLC measurements, canned salmon contains high levels whereas broiled chicken contains low levels (Hull 2012).

To further illustrate the point, Kellogg's Corn Flakes have either 70 kU of AGE per 30 gram serving (ELISA) or 1.6 mg per 30 gram serving size (UPLC). Again, this equates to a *very*

low measurement for corn flakes compared to most of the foods in the ELISA-based food AGE database and a high measurement of AGE compared to most foods in the UPLC-based food AGE database, exceeding that of braised steak, battered cod and pork sausage but not of whole milk yogurt (Goldberg 2004; Uribarri 2010; Hull 2012). This picture is confusing and needs to be rectified. A recent study comparing the use of UPLC, ELISA and another method to determine the CML content of known samples of glycated bovine serum albumin found good correlation between the various methods; however the r^2 value was just 0.75 for UPLC and ELISA. (Srey 2010) Foods were not tested in this study, thus more work needs to be done to evaluate whether these methods are equally valid in testing CML content of a variety of foods.

Measurement of serum and plasma AGEs in human subjects is less difficult and less controversial. CML is the favorite AGE for measurement, and a few kits are available. However, reporting of data is not in standard units.

Serum AGEs have been reported in the range of 8.5 ± 0.9 Units/ml in men less than age 45, 9.9 ± 1.5 in men older than 60, 7.9 ± 0.7 in women less than age 45 and 10.7 ± 1.1 in women older than 60 in a study of 172 healthy individuals (Uribarri, Negrean 2007). In a group of 60 healthy women and women with polycystic ovarian syndrome (PCOS), healthy women had AGE levels of 5.85 ± 0.89 Units (U)/ml, and women with PCOS had higher levels: 8.7 ± 1.65 U/ml (Diamanti-Kandarakis 2009). Yamagishi (2006), using a different CML antibody for ELISA, has reported serum CML concentrations of 4.1 ± 0.7 U/ml in men and 4.1 ± 0.9 U/ml among 184 healthy participants.

In the Italian In-CHIANTI study CML was reported in nanograms (ng)/ml, and participants with the slowest walking speeds had reported plasma CML levels of 375 ng/ml compared to 343 ng/ml for those with the fastest speeds (Semba, Bandanelli 2010). Using the

same assay, a separate study reported CML levels of 344 ng/ml for healthy participants and 390 ng/ml for participants with chronic kidney disease (Semba 2009). Reporting values in standard units might help establish norms for AGEs and make it more useful as a biomarker for inflammation.

ENDOGENOUS AGEs

AGEs have both endogenous and dietary origins and are thought to contribute to oxidative stress within the body. Endogenous AGE formation occurs slowly but may be up-regulated in conditions of metabolic stress such as sepsis and insulin resistance and in disease states such as diabetes and chronic kidney disease. Hyperglycemia may also lead to up-regulation of pathways other than glycolysis including the polyol pathway, which increases AGE production. Activation of protein kinase C could also stimulate the hexosamine pathway, increasing endogenous AGE. Furthermore, an increase in reactive dicarbonyls and reduced detoxification by the glyoxalase system is thought to lead to a state of carbonyl stress, which may increase endogenous AGEs. (Barlovic 2011)

EXOGENOUS AGEs

Because AGEs are glycated molecules, one might expect that foods rich in carbohydrates would be important dietary sources. In fact exogenous AGE consumption typically increases in high fat diets and when high temperature cooking methods such as deep-frying, broiling, roasting and grilling are used. This is particularly true for high protein products (Semba, Nicklett 2010). However, CML tables produced by Hull, which employ UPLC, also indicate the presence of increased AGEs in high carbohydrate foods (Hull 2012).

Whether dietary AGEs contribute significantly to deleterious health effects is a topic of active investigation. They were once considered insignificant due to early research showing that

small amounts of dietary AGE were absorbed in rats, primarily in the large intestine, with only about 1.5% being found in the liver after absorption (Sgarbieri 1973). A 1997 study in humans found only about 10% of dietary AGEs were absorbed. However, in this study, in persons with normal kidney function, approximately 30% of ingested AGEs were found to be excreted in the urine; whereas, for those with impaired kidney function, as estimated by glomerular filtration rate, as little as five percent of ingested AGE was excreted in the urine. A high AGE diet was also found to result in significant elevations in serum AGE post feeding despite low absorption (Koschinsky 1997).

In an *in vitro* experimental model assessing diet derived AGEs' effects on glutathione (GSH) and GSH peroxidase (GPx) (indicators of oxidative stress), on human umbilical vein endothelial cells (HUVECs), it was reported that AGEs depleted GSH and increased GPx activity (Cai 2002). The authors concluded that prior to absorption, food-derived AGEs contain carbonyl species, which may induce oxidative damage.

The larger studies which have attempted to create CML food databases are ELISA based: Goldberg 2004 and the follow-up, Uribarri 2010. A limitation of the Goldberg study was that the methods section did not describe whether multiple samples/trials were performed in order to determine AGE. AGEs were reported to be lowest in carbohydrate foods with the lowest levels within this group being found in milk, followed by vegetables and fruits. Broiled beef, and chicken, oils heated to high levels and roasted nuts were among the foods highest in CML. These data have been criticized in part because of the finding that oils were found to be high in CML. Because oils should contain no lysine, some question how they could also be rich in CML. However in a later publication, the researchers hypothesized that extraction and purification procedures accounted for this finding (Uribarri 2010). Elsewhere the same group of researchers

analyzed food records from healthy participants and found mean daily AGE intake to be about 14,780 +/- 680 kU AGE (Uribarri 2007; Uribarri 2010) compared to an earlier report of average intakes of 16,000 ± 5000 kU AGE (Goldberg 2004).

A study of a variety of common foods published in 2009 found levels of CML to vary from as low as 0.3 mg/kg of raw milk and 0.35 mg/kg of skim milk to as high as 46.1 mg/kg of whole meal bread crust (compared to just 4.45 mg/kg of bread crumb) (Assar 2009). Commercial breakfast cereals and ice creams also appear to be sources of AGE (Delgado-Andrade 2006; Drusch 1999). An evaluation of a variety of processing methods for nuts and seeds found that CML production was increased by roasting methods. Thus consumption of cooked foods compared to raw foods increases AGE ingestion (Yaacoub 2008).

Intermediate products or AGE-forming metabolites include methylglyoxl (MG) and glyoxal (GO). In a study evaluating the MG and GO content of soft drinks the trend was for products containing high fructose corn syrup to be relatively high in MG whereas diet drinks were not (Tan 2008). However, another analysis of AGEs in soft drinks employing ELISA found that AGE levels in soft drinks did not correlate with sugar content as long as protein and heat were not present. In fact drinks with caramel additives such as Coke Classic or Diet Coke were found to contain 8500 and 9500 units/cup compared to 475 units/cup in Sprite, 600 in orange juice and around 2000 in coffee and tea (Koschinsky 1997).

METABOLISM AND PATHOPHYSIOLOGY OF AGES

Enzymatic Disposal and Mechanistic Action

The body has a number of ways for degrading AGEs including the degradative enzymes glyoxalase I and II, which degrade and detoxify the pre-AGEs MG and GO along with aldose reductase and carbonyl reductase, which prevent formation of AGEs. Circulating proteins such

as lysozyme, defensins and lactoferrin may also bind AGEs in circulation, preventing them from binding elsewhere within the cells (Vlassara 2008). However, degradation may not always be rapid enough to keep up with production as in cases of hyperglycemia or severe oxidative stress. In addition, in kidney disease the body appears to decrease its capacity to degrade and excrete AGE to sufficient levels (Koschinsky 1997).

There are also two types of cell surface AGE receptors, one which binds AGE and initiates cell activation and another which binds, internalizes and degrades AGE. The RAGE receptor for AGE is thought to initiate oxidative stress whereas the AGERs 1, 3, and CD35 receptors help to mediate degradation of AGE (Vlassara 2008). Damage caused by AGEs is thought to result from activation of nuclear factor kappa beta (NF-κβ) which leads to up-regulation of genes for cytokines, growth factors and cell adhesion molecules (Kalousova 2005).

Brownlee (2001) described ways in which AGEs may damage target cells leading to diabetic complications. Three primary means of damage may be: modification of cellular proteins by glycation leading to loss of function, extracellular proteins or other molecules modified by AGE might interact differently with their receptors, and receptor-mediated AGE oxidative processes may also cause cellular damage.

In fact most mechanistic explanations of how AGE may cause oxidative damage suggest that AGEs exert influence via receptors. RAGE receptors may be expressed on the surface of a variety of cells including neurons, smooth muscle cell monocytes, endothelial cells, fibroblasts and more (Kalousova 2005). The RAGE receptor may then activate signal transduction pathways including extracellular signal-related kinase (ERK), mitogen activated protein kinase (p38 ^{MAPK}), c-jun N terminal kinases (JNK) and NF-κβ. Thus, transcription of genes may be stimulated which will up-regulate production of growth factors such as tumor necrosis factor (TNF-α), inter-

leukin 1 (IL-1) and insulin like growth factor-1 (IGF-1) and adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecules (VCAM-1). In addition, it is suggested that DNA may undergo glycation. Thus, AGE modification of DNA could affect regulatory and epigenetic processes at the DNA level (Ramasamy 2005). In addition, RAGE may be differentially spliced forming endogenous secretory RAGE (esRAGE). RAGE may also be cleaved from the cellular surface. The pool of both differentially-spliced and cleaved RAGE is known as soluble RAGE (sRAGE) which may act as a decoy receptor aiding in secretion of AGE (Barlovic 2011).

Pharmacokinetics

As discussed earlier under exogenous AGEs, one major study has attempted to answer questions regarding the pharmacokinetics of AGEs. In it, 43 subjects, (38 male and 5 female) including 38 with diabetes and 5 healthy participants were given high AGE test meals, which was followed by 48 hours of post meal evaluation. Urine samples were collected 48 hours after the test meal. Changes in serum and urine AGE kinetics were calculated and plotted as the area under the curve (AUC) (Koschinsky 1997). Participants consumed differing quantities of AGE, but there was a correlation between the amount consumed and the area under the curve (elevation in serum AGE). Only 10% of the AGEs ingested were accounted for in serum in this study. Only one-third of the AGEs detected in serum were detected in the urine. Thus, what happened to the other two-thirds was not explained by this study. (Koschinsky 1997)

Other studies related to pharmacokinetics of CML have primarily been done in animals (Somoza 2006; Tessier 2012) with a few exceptions. Birlouez-Aragon et. al. reported higher urinary excretion of CML in participants fed a high AGE diet; however labeled products were not fed, so the relationship was correlational only (Birlouez Aragon 2010).

PATHOPHYSIOLOGY

Healthy Individuals

There is a paucity of human studies involving healthy adults and AGE. Uribarri and co-investigators correlated calculated dietary AGE content and serum AGE in 90 healthy subjects and found increases in CRP with both dietary AGE and serum AGE (Uribarri 2005). In five participants taken from this group of 90, short term dietary AGE restriction decreased serum AGE levels by 30-40 percent. Based on this single study it is possible to say only that dietary AGE may significantly impact AGE levels even in healthy individuals in spite of low absorption.

Birlouez-Aragon also conducted an AGE feeding study involving healthy individuals with average BMI of 21.8 kg/m^2 (range 18-26.9) and found that after four weeks of feeding a steamed diet (low in CML) versus a conventional diet (comparatively higher in CML), participants on the steamed diet had 5% lower plasma total cholesterol, 10% lower HDL, and 9% lower triglycerides (Birlouez Aragon 2010). However, it should be pointed out that the participants consuming the steamed diet (who were fed *ab lib*) consumed significantly less energy, significantly less total fat, and more vitamin C than the participants consuming the standard diet, thus this data is somewhat confounded.

Obesity

AGEs may play a more prominent role in the health of obese individuals. The literature suggests that AGEs are involved in inflammatory processes, which are often increased in obesity (Ramasamy 2005; Brownlee 2001). The extent to which elevated circulating AGEs are the cause or symptom of disease and how AGEs, RAGE and sRAGE may be influenced by factors such as body fatness and lifestyle is not clear.

In several studies in older adults, fatness and sRAGE appear to be linked. Koyama et. al. reported an inverse correlation of components of the metabolic syndrome with sRAGE. Components demonstrating this inverse relationship included body mass index, TG, HbA1c and insulin resistance, which were all inversely linked with sRAGE in both diabetic and non-diabetic participants (Koyama 2005). Another Japanese group also found sRAGE to be inversely correlated with BMI, waist circumference, AGE intake and alcohol intake (Yamagishi 2006). Norata et. al. also reported that sRAGE was inversely correlated with BMI, waist/hip circumference and fasting glucose with a positive correlation to apolipoprotein A-1 (Norata 2009). Thus, the link between sRAGE and fatness in older adults seems to be established based on several studies done in separate populations.

In studies relating AGEs to BMI, the correlation is usually direct. Semba et. al. used DEXA to establish body fatness and found that total fat mass, truncal fat mass and appendicular fat mass were each correlated with serum CML when controlled for age, sex, BMI, blood pressure, TG, HDL and renal function. This suggests that body fat affects CML and possibly other AGEs perhaps because AGEs may be stored in fatty tissue (Semba 2011). Diamanti-Kandarakis et. al. also found a correlation between AGEs and BMI (Diamanti-Kandarakis 2009), as did a study by Yamagishi et. al. (Yamagishi 2006). However, in a study of obese children and adolescents, levels of AGEs were actually lower in the obese children compared to controls (Sebekova 2009). Given more limited data in this area, the link between AGEs and fatness is suggestive but not confirmed.

Finally Yamagishi et. al. identified a correlation between sRAGE and AGE. However, they found a positive correlation between the two. This seems counter-intuitive given research suggesting that AGEs increase with increasing BMI whereas the link is opposite for sRAGE. This

group of researchers hypothesized that sRAGE may reflect tissue RAGE expression and that sRAGE may increase along with AGE in order to mount a counter-defense (Yamagishi 2006).

In the only study of weight loss and AGEs in obese subjects, Gugliucci et. al. treated 37 Japanese participants (30 females, 7 males) with a low calorie diet (1200 kcals/5020 KJ daily) for 2 months. Participants reduced their BMI, waist circumference and TG but also reduced serum AGEs by 7.2%. Whether the reduction in AGEs was due to decreased fat mass, a reduction in glycation due to caloric restriction, or a decreased intake of dietary AGEs was not assessed. (Gugliucci 2009)

Diabetes and Diabetes-Related Cardiovascular Disease

The link between serum AGE and inflammatory stress in diabetes is comparatively strong. In a study by Tan (2004), serum AGEs were significantly increased in persons with diabetes, and CRP was highly correlated with AGEs. Regression analysis demonstrated that BMI, interleukin-6 (IL-6) and CRP were determinants of AGEs (Tan 2004). An earlier study also by Tan found significant increases in serum AGE in persons with diabetes and found AGEs to be correlated with endothelium-dependent vasodilation (Tan 2002). In addition, dietary AGEs may result in prolonged increases in serum AGEs in persons with diabetes. Koschinsky et. al. evaluated the effect of a single meal containing egg white cooked with or without fructose (with=AGE; without=control) in 38 participants with type 2 diabetes. This study first showed that not only was serum AGE increased with dietary consumption but also that AGE levels remained elevated in persons with both diabetes and severe renal disease for more than 48 hours after ingestion (Koschinsky 1997).

Studies involving diabetic complications related to AGEs have shown that AGEs are increased in vessels of the retina and in renal glomeruli in persons with diabetes (Brownlee 2001).

Animal based studies have also demonstrated fewer diabetic complications, especially microvascular complications, in mice fed reduced AGE diets. Follow-up multicenter trials of an AGE inhibitor (aminoguanidine) demonstrated reduced diabetic retinopathy (Brownlee 2001). While this drug did not progress to clinical trials, other potential AGE drugs include AGE breakers such as ALT 711 (Friedja 2012).

Studies relating to macrovascular complications of diabetes also indicate a role for AGEs. Patients with diabetes randomized to a low AGE diet were found to have less glycated serum LDL and oxidized serum LDL compared to those fed the standard diet (Cai 2002). In the Diabetes Control and Complications Trial, oxidized and glycated LDL were found to correlate well with duration of diabetes, BMI, and lipid levels (Lopes-Virella 2011). Serum AGEs were found to correlate with triglyceride levels in a study of diabetes-related cardiovascular disease, and those with the highest AGEs had the most adverse lipid profiles (Chang 2011).

While mechanisms for damage in diabetes have usually been related to signaling and inflammatory processes as previously described, one study involving both cells and mice with diabetes found that AGEs were acutely toxic to pancreatic beta cells, causing loss of manganese superoxide dismutase (SOD) and increased glucose uptake. In addition, rats fed high AGE diets had progressive insulin secretory defects and beta cell death (Coughlan 2011).

Studies which have attempted to manipulate the diet of persons with diabetes to include fewer AGEs have demonstrated positive results. Negrean et. al. fed persons with type 2 diabetes isocaloric diets with identical ingredients but which were prepared using different cooking methods in order to obtain high and low AGE test meals followed by measurement of endothelial function using flow-mediated dilation (FMD) and Laser-Doppler flowometry. FMD was found to decrease significantly more after the high AGE meal (a decrease which was 1.5 times greater than

the low AGE meal change). AGE did not affect glucose, triacylglycerol levels or insulin. No significant changes in inflammatory markers were found (Negrean 2007, Stirban 2008).

A separate report of a similar study by the same group of researchers demonstrated increased serum AGE and increased glycated LDL in 11 diabetic subjects fed a high AGE diet in a two-week crossover study. Thirteen participants in a longer 6 week low or high AGE intervention also were noted to have increased glycated LDL while on the high AGE diet (Vlassara 2002).

In feeding stable diabetic participants and healthy participants an oral AGE challenge beverage with no carbohydrate or lipid, Uribarri et. al, found that maximal arterial dilation after ischemia decreased significantly with no changes in glucose or VCAM-1. This decrease in FMD is thought to be indicative of impaired endothelial function resulting from the AGE bolus (Uribarri, Negrean 2007).

Each study seems to indicate a possible positive impact of a low-AGE diet on diabetes-related cardiovascular diseases. However, even though many studies in the field of diabetes have been done with regard to AGEs, the evidence is regarded as insufficient to warrant changes in dietary advice to persons with diabetes (*Diabetes Care* 2012).

Kidney Disease

AGEs are thought by some to damage nephrons by altering the structure and function of proteins and by injuring cells (Uribarri 2006) due to changes in cellular adhesion molecules and inhibition of processes leading to the production of type IV collagen and laminin fibers (Uribarri 2006). Serum AGEs correlate with inflammatory markers and are inversely associated with creatinine clearance (Vlassara 2009). Proof that AGEs damage renal function is currently lacking, but suggestive data is available.

The impact of dietary AGEs on kidney function has been evaluated in overweight and obese adult males randomized to two weeks on a low and high AGE diet (Harcourt 2011). In this study fat was held constant at 30% with AGE being the primary variable, and AGE was calculated based on the Goldberg values (2004). The researchers found that albumin to creatinine ratios were improved following the low AGE diet. In addition plasma AGE increased following high AGE consumption. Some drawbacks to this study design include likely differences in type of fat in the study due to the dietary approach used.

In a large study of older community-dwelling adults, CML was associated with chronic kidney disease and glomerular filtration rate (GFR), the primary indicator of CKD. This remained true at 3 and 6 year follow-up. While this study shows CML to possibly be a good indicator of renal function, it does not prove that dietary CML negatively impacts GFR (Semba 2009). Similarly in the Baltimore Longitudinal Study of Aging in which 750 adults were followed for 52 years, serum CML was associated with CKD ($p=0.003$) and decreasing GFR (Semba, Fink 2010).

Studies in animals have demonstrated that aminoguanidine (an AGE inhibitor) decreases kidney disease in aging rats (Vlassara 2009; Li 1996). In addition a life-long restriction of dietary AGEs in mice decreased kidney lesions in aging mice (Vlassara 2009; Cai 2008).

However, Schwedler et. al. found that elevated serum AGEs were not related to mortality in patients on hemodialysis (HD). CML and AGE were definitely lower in healthy controls compared to those on HD, but mortality was not associated with CML (Schwedler 2002).

A fairly scathing critique of the above literature in kidney disease and AGEs research was recently written by Piroddi et. al. This group of researchers measured CML in serum using chemiluminescent detection and immuno-blots and pentosidine using reverse-phase HPLC

analysis (Piroddi 2011). CML and pentosidine levels were increased in CKD and HD patients, but intake of CML was lower in participants with CKD or HD. Piroddi et. al. reported a negative correlation between CML intake and plasma CML and a positive correlation of CML and protein intake. They concluded that CML in the plasma does not correlate with dietary intake and should not be restricted in healthy individuals or those on HD. They also claimed that the CML epitope measured by the assay used by Vlassara, Semba and others at Mt. Sinai is present in low abundance in human serum and in food (Piroddi 2011).

Cardiovascular Disease

An animal model developed by Ueno and Koyama has sought to determine whether RAGE is linked to progression of atherosclerosis (Ueno 2010). Variations of RAGE and apolipoprotein E (ApeE) knockout mice were produced and randomly assigned to atherogenic diet and standard chow diet from 6 weeks to 20 weeks of age. The $ApoE^{-/-}RAGE^{-/-}$ mice fed with either diet had decreased plaques even though no differences in cholesterol or glucose were observed. The $ApoE^{-/-}RAGE^{-/-}$ mice also gained significantly less body weight despite no difference in food intake. Thus, it appears that mice deficient in RAGE have improved body fatness and less atherogenesis, an intriguing finding should it also be found to be true in humans. (Ueno 2010)

Another group of researchers identified a decrease in leptin after high AGE feeding in 20 in-patients with type 2 diabetes. They also found decreases in adiponectin and increases in VCAM-1 and e-selectin (Stirban 2008). The same group did a separate study involving 13 persons with type 2 diabetes and found that a high AGE test meal significantly impaired macrovascular FMD (Stirban 2006). The findings in the animal study together with the studies

previously discussed with regard to cardiovascular disease related to diabetes (Cai 2002, Lopes-Virella 2011, Chang 2011, Vlassara 2002, Negrean 2007, Stirban 2008), indicate a possible association between foods high in AGE and impacts on oxidized LDL, glycated LDL, and endothelial dysfunction, all indicators of atherogenesis.

Population based studies have also indicated a possible role for AGEs in cardiovascular disease and mortality. Kilhovd et. al. determined in a Finnish population of persons with and without diabetes that increased serum AGE predicted total and cardiovascular disease-related mortality in women but not men (Kilhovd 1999, 2005, 2007). Similarly, Nin et. al. found plasma AGE (CML and pentosidine) to be linked to fatal and non-fatal cardiovascular disease in 339 patients with type 1 diabetes (Nin 2011). Kiuchi et. al also identified that AGE levels increased with severity of cardiovascular disease in a Japanese population with diabetes (Kiuchi 2001).

CONCLUSIONS

Much has yet to be done in AGEs research. We need to better understand the pharmacokinetics of AGEs. With so little being excreted in the urine or discovered deposited in tissues, what is the fate of the remainder of ingested AGEs? Are there important differences in the influence of AGEs in the body based on disease state and age? Do different AGEs interact with receptors differently with different results? More fundamentally, which AGE is the most important to measure, and can we decide as a scientific community on an ideal way of quantifying AGE in the body and in foods?

Another incomplete area of research includes the interaction between dietary macronutrient composition and serum AGE and sRAGE. Much has been made of the importance of low fat diet in lowering risk of cardiovascular disease. However, cooking methods recommended for lowering fat intake such as broiling, grilling and roasting tend to increase

concentrations of AGEs in foods. Current research suggests that AGEs increase oxidative and carbonyl stress, decrease cardiovascular endothelial dysfunction, and contribute to cellular damage related to diabetes and chronic kidney disease. In addition increased body fat and body weight are associated with higher levels of AGE and decreased levels of an important receptor for AGE (sRAGE). Since low-fat diet is often recommended for weight control in obese and overweight persons, it is important to determine to what extent a low fat diet using these commonly advised cooking methods may contribute to AGE load and whether such a low-fat diet may negatively affect inflammatory cytokines thought to be influenced by AGE.

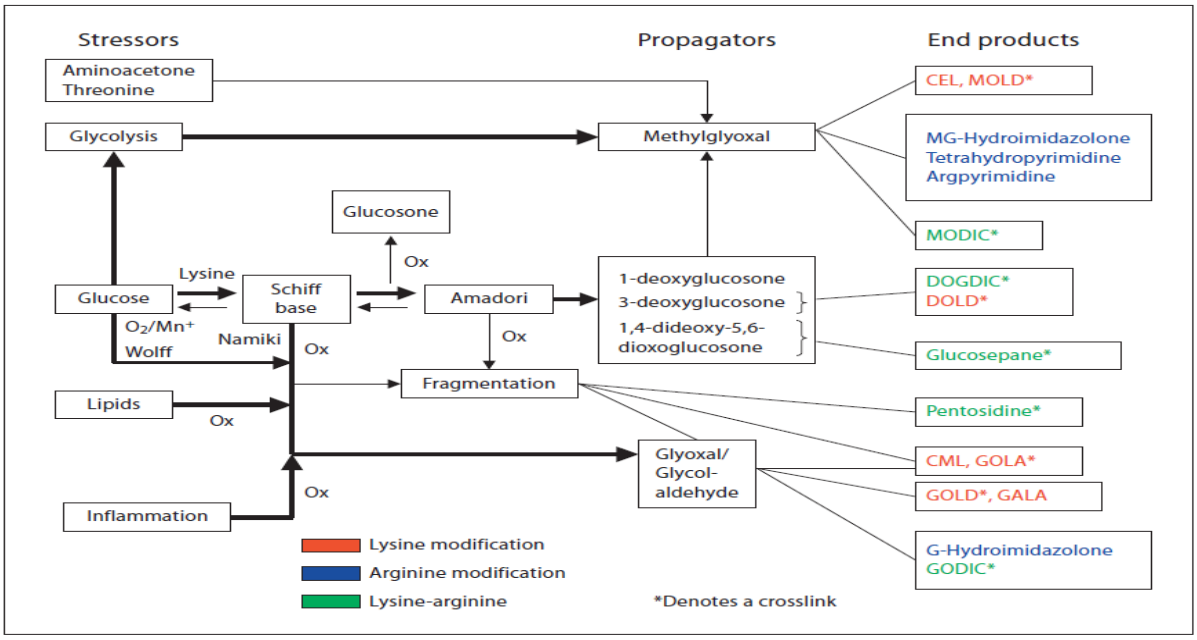

Fig. 1. Conceptual scheme of the Maillard reaction summarizing the major known glycation pathways involved in arterial stiffness. CEL = Carboxyethyl-lysine; CML = carboxymethyl-lysine; DOGDIC = 3-deoxyglucosone-derived imidazolium cross-link; DOLD = 3-deoxyglucosone-lysine dimer; G = glyoxal; GALA = glycolic acid-lysine-amide; GODIC = glyoxal-derived imidazolium cross-link; GOLA = glyoxal-lysine-amide; GOLD = glyoxal-lysine dimer; MG = methylglyoxal; MOLD = methylglyoxal-lysine dimer; MODIC = methylglyoxal-derived imidazolium cross-link.

Figure 5.1.* Formation of AGEs.

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CHAPTER VI

HIGH-FAT, HIGH-ADVANCED GLYCATION END PRODUCTS DIET PROMOTES RISE IN SERUM N-EPSILON-CARBOXYMETHYLLYSINE

A Paper to be Submitted for Publication

ABSTRACT

The Western diet, which is high in simple carbohydrates and fat and low in fruits and vegetables, favors inflammation. However, the cause of this inflammation is controversial. Some research suggests that advanced glycation end products (AGEs), compounds produced when foods are heat treated at high temperatures may play a role. The purpose of this study was to determine whether macronutrient content (low-fat versus high fat diet) influences an indicator of AGEs: n-epsilon carboxy-methyl-lysine (CML) in the presence of a high AGE diet and whether the soluble receptor for AGEs (sRAGE) increases in response to a dietary AGE load. A pattern toward decrease in CML was found following consumption of a low-fat high AGE diet ($p=0.12$), and a pattern indicative of a rise in CML occurred following consumption of a high fat high AGE diet ($p=0.15$). The mean change in CML between the two diets approached, but did not reach significance ($p=0.08$). sRAGE decreased in the hours after consumption of either high AGE meal ($p=0.01$). CRP and adiponectin were not affected by either test diet. These findings suggest that dietary AGE may not be very important in influencing inflammation or even serum CML in healthy adults without diabetes.

INTRODUCTION

Western diet as it exists today favors inflammation and development of chronic diseases. Theories for the mechanism behind this observational finding include dietary imbalance of n-3

and n-6 fatty acids, consumption of high fat diet, inadequate intake of micronutrients and plant foods, excessive intake of refined or processed foods, and an imbalance in consumption of dietary acid and base (Cordain 2005). A less well known theory purports that cooking foods produces advanced glycation end products (AGEs), compounds formed when sugars react with amino acids or other substances forming glycosylated molecules, which are thought to favor oxidative stress when absorbed (Kalousova 2005; Vlassara 2011; Semba 2010, Barlovic 2011). AGEs include pentosidine, n-epsilon-carboyxmethyllysine (CML), hydroimidazolone, furosine, glucosepane, and many others. CML is the most widely studied and is regarded as a good proxy measurement of AGE load in the body based on early studies (Cai 2008; Brownlee 2001; Requena 1997).

Advanced glycation end products are produced very slowly within biological systems as blood glucose interacts with body proteins, but AGEs may also enter the body via dietary consumption, although only about 10% of dietary AGEs are absorbed (Sgarbieri 1973, Koschinsky 1997). AGEs may increase oxidative stress via affinity for the receptor for AGE (RAGE), which initiates a cascade of events leading to decreased cardiovascular endothelial dysfunction and possible diabetic complications (Vlassara 2011). The soluble receptor for AGE (sRAGE) is thought to act as a decoy receptor, aiding the body removal AGEs (Raucci 2008; Kalea 2011).

Several studies in food have demonstrated that dietary AGEs are present in high concentrations in foods exposed to high temperature cooking methods, such as deep-frying, broiling, roasting, baking and grilling (Uribarri 2010). Additionally, foods that are high in protein and high in fat which are cooked using these methods tend to be particularly high in AGEs (Semba 2010). However, there is little consensus about appropriate measurement techniques for

quantifying AGE in foods and whether endogenous or dietary AGE found in a heat-processed Western diet may negatively impact inflammation (Ames 2008, Delgado-Andrade 2007, Drusch 1999, Assar 2009, Vlassara 2011). Despite low levels of absorption, several studies have found decreased serum CML in participants (both human and animal) following reduced AGE diets (Koschinsky 1997, Cai 2002, Vlassara 2002, Uribarri 2005, Sandu 2005, Diamani-Kandarakis 2006, Negrean 2007, Stirban 2008, Cai 2008, Uribarri 2007, Vlassara 2009, Harcourt 2011, Uribarri 2011). In addition, a study by Tan et. al. (2004) found a correlation between dietary AGEs and C-reactive protein (CRP), a prominent indicator of inflammation. However, no study has been done to compare the effect of consumption of a high-fat diet versus a low-fat diet when both are elevated in AGEs on serum CML and sRAGE. Currently, the effect of dietary AGE on serum AGE is not clear since the publication of a study by Semba et. al. in early 2012 which found no relationship between dietary records detailing AGE intake and serum AGE.

Current research suggests that AGEs increase oxidative and carbonyl stress, which may cause endothelial dysfunction, and contribute to cellular damage related to diabetes and chronic kidney disease. Additionally, increased body fat and body weight are associated with higher levels of AGE and decreased levels of an important receptor for AGE (sRAGE). Since low fat diet is often recommended for weight control in obese and overweight persons, it is important to determine whether a low fat diet prepared using cooking methods such as baking, broiling, roasting, and toasting contributes to AGE load and negatively affects inflammatory cytokines thought to be influenced by AGE.

The purpose of this study was to determine if a low fat diet cooked using high heat methods has equivalent effects on serum AGE and inflammatory cytokines when compared to a high fat diet cooked using similar high heat cooking methods.

METHODS

This study was approved by the Texas Woman's University Institutional Review Board's Human Subjects Committee, and each participant provided written consent prior to participation in the study.

Non-smoking participants age 20-45 with BMI of 27-35 (overweight or class 1 obesity) were recruited from two university campuses and from the wider community. Participants were instructed to adhere to their usual level of activity during the study period, and information on any supplements or medications taken by participants was obtained. Exclusion criteria included self-reported hypertension, dyslipidemia, kidney disease, diabetes, heart disease, and metabolic disease. Smokers were also excluded. Participants were advised to take no supplements for at least one week prior to the study and to avoid anti-inflammatory drugs. Participants were also screened for medications which could affect blood glucose, lipids or inflammation and excluded if any of these were identified.

Recruitment methods included electronic announcements, print flyers, and communication with local doctors' offices, gyms, clubs and community organizations. The goal for recruitment was 15 men and 15 women. Nine men (Age: 34.2 years ± 9.8; BMI: 29.6 kg/m^2 ± 2.7) and 10 women (Age: 28.6 years ± 5.5 years; BMI: 31.3 kg/m^2 ± 2.9) were recruited. One female participant was excluded due to extremely high CRP, lipid, and CML measurements. Thus, 18 participants (9 males, 9 females) were included in the study.

Anthropometric, Biochemical Measurements and Study Protocol

Using a cross-over study design, all study participants consumed both study diets. Diet A was a low-fat, high AGE diet (LFHA). Diet B was a high fat, high AGE diet (HFHA). On day one of the study, participants reported to the lab for anthropometric measurements. A portable stadiometer was used to measure height to the nearest one-quarter inch, and a beam scale was used to measure weight to the nearest 0.5 pound. Height and weight measurements were converted to centimeters and kilograms for reporting, and body mass index (BMI) was calculated (kg/m^2). Waist circumference was measured with a non-stretch tape measure to the nearest 0.5 cm at the narrowest part between the lower costal (10^{th}) rib border and iliac crest, using the navel as a marker. Percentage body fat was measured using bioelectrical impedance analysis (BIA) (Tanita electronic scale). Anthropometric measurements were taken again immediately prior to the second dietary intervention period by the same personnel.

Five blood draws were conducted during each phase of the study. The first blood draw was done at baseline during the first trip to the lab with follow-up blood draws to include a 1 hour, 2 hour and 3 hour post-prandial draws after consumption of the first test breakfast meal. After completion of the 3 hour post-prandial blood draw, participants consumed lunch. They consumed an afternoon snack, dinner and a bedtime final snack over the course of the remainder of the day. Participants were allowed to come and go during each study day so long as they were on-time for blood draws. They returned to the lab the next morning for another fasting draw.

After a two week wash-out period, participants returned to campus for the same procedure using the alternate study diet not yet consumed. Anthropometric measurements were done immediately prior to the second dietary intervention period by the same

personnel. The same protocol was used for both dietary interventions. Each blood draw was be performed by a trained nurse or certified phlebotomist. The order of meals was switched at random for test groups beginning the trial so that an almost equal number of subjects completed the low-fat diet first (n=10) compared to those consuming the high fat diet first (n=8).

Diet Intervention

Participants received two isocaloric study diets: one containing 20% of calories from fat, the other with 41% of calories from fat. The fatty acid distribution of both diets was 1:2:1 (MUFA:PUFA:saturated fat). High AGE cooking methods were used for each: broiling, roasting, baking, and frying. See Table 6.1.

Table 6.1. Macronutrient and AGE Content of Study Diets: Low-Fat, High AGE and High Fat, High-AGE Diet

Meal	Low-Fat Diet	High Fat Diet
Breakfast	30 g Rice Krispies cereal [600]* 250 ml Fat free milk [4] 25 g (1 slice) whole wheat toast [21] w/2.5 g Smart Balance [156] 90 g smoked deli ham [2114] 85 g banana [8] 120 ml 100% apple juice [3] Total AGE Meal: [2906]	30 g Life cereal [394] 250 ml Whole milk [12] 25 g (1 slice) whole wheat toast [21] w/5 g Smart Balance [311] Bacon, microwaved (20 g) [1805] 85 g banana [8] 120 ml 100% apple juice [3] Total AGE Meal: [2554]
Lunch	Toasted cheese sandwich: 25 g x 2 whole wheat bread [42] 30 g reduced fat cheddar cheese [737] 2.5 g Smart Balance [156] 50 g carrot sticks [5] 240 ml chicken noodle soup [4] 100 g baked apples with 15 ml honey [46] 250 ml Coke [7] Total AGE Meal: [997]	Toasted cheese sandwich: 25 g x 2 whole wheat bread [42] 30 g regular cheddar cheese [1657] 5 g Smart Balance [311] 50 g carrot sticks [5] 240 ml chicken noodle soup [4] 100 g apples-no skin [13] 250 ml diet Coke [3] Total AGE Meal: [2035]
Dinner	90 g broiled chicken breast [5245] 100 g Grilled California veggies (broccoli, carrots, cauliflower) [226] (1.25 tsp corn oil) [150] 100g roasted potatoes [218] 35 g whole grain roll [29] 2.5 g Smart Balance [156] 60 g Frozen fruit bar [11] 120 ml orange juice [7] 360 ml Diet Coke (12 oz can) [4] Total AGE Meal: [6046]	90 g pan fried chicken breast [4444] 100 g Grilled California veggies (broccoli, carrots, cauliflower) [226] (1.25 tsp corn oil) [150] 50 g fried potatoes [347] 35 g whole grain roll [29] w/5 g Smart Balance [311] 60 g frozen fruit bar [11] 360 ml Diet Coke (12 oz can) [4] Total AGE Meal: [5522]
Snack 1	30 g Roasted cashews (1 oz) [2942] 250 ml fat-free milk [4] With 1 Tbsp syrup (chocolate or strawberry) Total AGE Snack: [2946]	30 g Roasted Almonds (1 oz) [1995] 250 ml whole milk [12] Total AGE Snack: [2007]
Snack 2	20 g cocktail peanuts [1667] 30 g Snyder's pretzel minis [537] 120 ml apple juice [3] Total AGE Snack: [2207]	28 g cocktail peanuts [2333] 30 g goldfish [653] Total AGE Snack: [2986]
Total AGE (kU)	2906+997+6046+2946+2207=15,102 KU (1.51×10^7)	2554+2035+5522+2007+2985=15,098 KU (1.51×10^7)
Total Calories	2249	2246
% Fat	20 % (50g) (0.7:1:0.6 SF:MUFA:PUFA)	41 % (104g) (0.9:1:0.5 SF:MUFA:PUFA)
Protein	108 g (19%)	103 g (18%)
Carb	356 g (62%)	235 g (41%)

*Numbers in [brackets] represent the calculated AGE value of the food item from tables in the article by Uribarri et. al. 2010.

Breakfast and lunch were consumed in the laboratory. Dinner, afternoon and bedtime snacks were consumed in the laboratory or boxed and given to the participant to be consumed at

home according to participant need. Dietary macronutrient content was calculated initially using Nutritionist Pro, then confirmed by proximate analysis of the diet by an independent laboratory (Pope Labs). Analysis of the diet sample is shown in Table 6.2. Participants were allowed to eat only the study foods during each one day intervention period.

Table 6.2: Comparison of Calculated Macronutrient Values for Test Diets and Experimentally Determined Values.

	Diet A (LF HA): 3.07 kg		**Diet B (HF HA): 3.37 kg**	
Category	Pope	Calculated	Pope	Calculated
Fat	53.7 g (24%)	50 g (20%)	78.2 g (36%)	104 g (41%)
Protein	0.52% N 99.7 g (20%)	108 (19%)	0.48% N 101.1 g (21%)	103 g (18%)
Carbohydrate	302g (60%)	356 g (62%)	213 g (44 %)	235 g (41%)
Calories	2018 kcals	2249 kcals	1956 kcals	2246 kcals

Biochemical and Food AGE Analysis

Blood samples were immediately put on ice, allowed to sit for 10-15 minutes to facilitate clotting, and centrifuged at 1200 rpm for 15 minutes. Sera were separated, aliquoted and stored at -80^{o}C. Sera were analyzed for glucose, triacylglycerol (TG), total cholesterol (TC), and high density lipoprotein (HDL) using commercially available kits (StanBio, Boerne, TX). HMW adiponectin was measured using enzyme linked immunoassay kits (ELISA) (Alpco, Salem, NH).

CML, CRP, and sRAGE were also measured using ELISA (Microcoat, Bernried, Germany; Alpco, Salem, NH; Biovendor, Karasek, Czech Republic).

Food samples were prepared using defined techniques (Goldberg 2004), then pureed, combined, weighed, and frozen. One-hundred gram samples from each diet were retained and sent for analysis of macronutrient content (Pope Labs). In our laboratory, food samples were weighed, dissolved in PBS and quantified after pre-digestion using proteinase K (Roche Laboratories) using CML ELISA kits (Bernried, Germany). Measured dietary AGEs values were different from the calculated values. AGE content was similar between diets, with the low-fat diet being somewhat higher in AGEs than the high-fat diet. (Table 6.3)

Table 6.3: Comparison of Measured AGEs to Calculated AGEs in Test Diets

	Low-Fat Diet	**High-Fat Diet**	**Low-Fat Breakfast**	**High Fat Breakfast**
Calculated AGE	15, 102 KU	15, 098 KU	2960 KU	2554 KU
Measured AGE Trial 1	16.9 mg	14.3 mg	5.1 mg (acid treated)	4.2 mg (acid treated)
Measured AGE Trial 2	19.7 mg	16.2 mg	7.4 mg (proteinase K treated)	9.2 mg (proteinase K treated)

Statistical Analyses

Potential numerical outliers were identified using box plots and 1.5 times the Q_1 to Q_3 interquartile range. Data were analyzed with and without outlier data for comparison. Pearson's correlations were performed for all sera and anthropometric parameters. Paired t-tests were used

to compare data from the LFHA and HFHA interventions. In addition ANOVA was used to compare variables based on weight class, sex, presence or absence of hyperlipidemia, and presence or absence of high levels of inflammation. SPSS version 19.0 for Windows was used to perform all statistical analyses including descriptive statistics, analysis of data for normality, and correlations.

RESULTS

Participants tolerated both the HFHA and LFHA study diets well and ate all of the study foods in most cases except one participant was unable to finish her bacon, consuming just 50%. One participant reported indigestion, probably due to consumption of diet cola, and another participant reported allergic symptoms following consumption of cashew nuts. Otherwise, both study diets were well tolerated.

There were no changes in weight, BMI or percent body fat over the course of the study. Men and women were not different with regard to any study variable with the exception of percentage body fat. As expected, men had lower percentage body fat (27.4% ± 4.4 compared to 41.5% ± 4.1 for women; p=0.0001). Data for males and females were grouped together for further analysis. Histograms and box plots were checked to evaluate normality of the samples and to check for outliers. Because the sample size was small, data that exceeded 1.5 times the Q_1 to Q_3 interquartile range was not automatically excluded from analysis. Instead only one participant's data was excluded for a single indicator: sRAGE. It was determined that because serum values were 10 times the mean for the other participants, this was a likely true outlier. This participant's sRAGE data was excluded.

Although participants were screened for dyslipidemia, half of the participants had elevated total cholesterol or triglycerides of which they were unaware. Thus, t-tests were done to determine if biochemical or anthropometric data varied based on presence or absence of elevated lipids. Significant differences were found only with regard to blood lipids and CRP between participants with and without abnormal blood lipids.

Among overweight (n=10) and obese participants (n=8), there were significant differences in change in HMW adiponectin following consumption of the LFHA diet (Overweight: -0.6±0.3; Class 1 Obesity: 0.5±0.6; p=0.02) and in CRP (Overweight: 0.01±0.7; Class 1 Obesity: -0.06±0.4; p=0.02). All other biochemical values did not differ between groups.

Participants were stratified based on level of CRP: low, medium, high. Percentage fat, HDL and glucose were different among these groups. Other indicators approached but did not reach significance.

CML levels following consumption of the LFHA breakfast meal rose from a fasting mean of 610 ng/ml ± 205 to a peak of 716 ± 471 at 2 hours post-prandial (Table 5), then decreased to 549 ± 179 at fasting the next day (p=0.12). The average *decrease* in CML from baseline to fasting the next day was 62 ng/ml ± 157 (p=0.12).

By comparison, following consumption of the HFHA breakfast meal, CML levels rose from an average of 523 ng/ml ± 178 to a peak of 642 ± 210 at 3 hours post-prandial (p=0.01), then increased (compared to baseline fasting) to 582 ± 232 (p=0.15) the next day. The average *increas*e in CML from baseline to fasting the next day was 54 ± 161 (p=0.15). (Table 4) The differences in change in CML from baseline to fasting the next day for the two study diets approached, but did not reach significance (p=0.08). However, the trend was for LFHA, which

was slightly higher in measured AGE, to show a decrease in CML, whereas the HFHA diet, which was similar but slightly lower in AGE to demonstrate an increase in CML. Using a paired t-test, area under the curve (AUC) for CML at 0, 1, 2 and 3 hours was found not to differ significantly between the two study diets (LFHA: 1932 ± 916; HFHA: 1727 ± 441). In fact, the AUC for CML for the two study diets was correlated significantly (R=0.54, p=0.02).

The raw data demonstrated great variability from person to person in patterns of change in CML over time. Seven participants fed the LFHA diet showed a slight decrease in CML post-prandially following ingestion. Eight showed a slight increase and three a large increase. Only one participant fed the HFHA diet showed a slight decrease in CML post-prandially following ingestion of the diet with seven participants having a moderate increase and 10 a large increase.

sRAGE levels following consumption of both the LFHA and HFHA breakfast meals decreased significantly from baseline to 1 to 2 to 3 hours post-prandially ($p<0.01$), then rose the next day, though not-significantly. Mean baseline sRAGE prior to the LFHA diet was 545 pg/ml ± 181 rising to 587 ± 294 (p=0.32) the next day. Mean baseline sRAGE prior to the HFHA diet was 534 ± 217 rising to 554 ± 182 the next day (p=0.22). (Table 4) AUC for sRAGE at 0, 1, 2 and 3 hours did not different significantly between LFHA and HFHA diets.

Other biochemical parameters changed little from baseline to fasting the next day; however, total cholesterol decreased significantly for participants consuming the HFHA diet (Baseline: 220 mg/dl ± 46; Post: 209 ±41; p=0.03).

Table 6.4. Selected Biochemical Values Grouped According to LFHA and HFHA Diet.

LFHA Diet			**HFHA Diet**		
	Value	**Change**		**Value**	**Change**
CML 0 hr (ng/ml)	610±205		**CML 0 hr (ng/ml)**	523±178	
CML 1 hr	615±280		**CML 1 hr**	577±176	
CML 2hr	716±471	-106±415(p=0.3)	**CML 2hr**	567±167	
CML 3hr	590±369		**CML 3 hr**	642±210	-114±201(p=0.01)*
CML Post	549±179	62±157 (p=0.12)	**CML Post**	582±232	-54±161(p=0.15)
sRAGE 0hr (pg/ml)	545±181		**sRAGE 0hr (pg/ml)**	534±217	
sRAGE 1hr	529±199		**sRAGE 1hr**	487±183	
sRAGE 2hr	486±207		**sRAGE 2hr**	482±165	
sRAGE 3hr	466±186	97±24 (p=0.01)*	**sRAGE 3hr**	466±176	87±21(p=0.01)*
sRAGE Post	587±294	79±97 (p=0.32)	**sRAGE Post**	554±182	70±87 (p=0.22)
HMW Adipo 0 hr (µg/ml)	1.8±0.4		**HMW Adipo 0 hr (µg/ml)**	1.8±0.4	
HMW Adipo Post	1.6±0.4		**HMW Adipo Post**	1.5±0.4	
CRP 0 hr (µg/ml)	1.7±0.4		**CRP 0 hr (µg/ml)**	1.6±0.4	
CRP Post	1.5±0.4		**CRP Post**	1.4±0.3	

DISCUSSION

This study is the first to compare consumption of a low-fat and high fat diet, each high in AGEs, on serum AGE, sRAGE and indicators of inflammation. While our study was limited by several factors, including small sample size, the presence of many participants with hyperlipidemia or dyslipidemia despite screening to identify healthy participants, and the short-term nature of the study, its findings are important in a few ways.

Even though both diets were calculated to be the same in AGE and used almost the same foods with fruit juices added to the low-fat diet in order to compensate for decreased calories due to less fat, the measured content of the LFHA diet appeared to be about 15% higher in AGE than the high fat diet. However, whereas consumption of the LFHA diet was associated with a non-significant decrease in serum CML with no rise in CRP or reduction in adiponectin, the HFHA diet was associated with a rise in serum CML but also with no rise in CRP or reduction in adiponectin.

These findings suggests that dietary AGE may be less significant than other factors in the diet such as phytochemicals, dietary fat, or other unidentified factors, which may inhibit AGEs or affect other oxidative processes. Whereas several studies have found negative physiological effects of single test meals rich in AGE, ours found no such effect. CRP and adiponectin were very stable in the presence of both diets (Koschinsky 1997, Diamanti Kandarakis 2006, Uribarri 2007, Stirban 2006). It is not clear why, with healthful fats included in both test diets, very similar micronutrient profiles, and almost identical foods being purchased whenever possible, there would be a decrease in CML in the presence of the LFHA diet and a rise in CML after consumption of the HFHA diet. This finding suggests that serum CML really is not very

significantly influenced by diet in healthy adults at all given such low absorption, so long as these adults do not have diabetes. In addition, sRAGE in this study did not appear to be a highly useful indicator of metabolic risk. CRP appears to be more sensitive, more stable, and less subject to dietary influences. This adds credence to the finding of Semba et. al. in 2012 in which using dietary records to estimate AGE intake did not correlate with serum AGE levels. In this study, intake of foods thought to be high in AGEs also did not correlate with serum CML.

This study by no means answers any questions in AGE research. It only provides one more data point to aid in answering the question: are heat-treated foods in the presence of balanced diet, harmful? Based on these results, our answer at this time would have to be that in the short term, they are not.

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APPENDIX A:

Institutional Review Board Approval Study I

TWU
TEXAS WOMAN'S UNIVERSITY
DENTON DALLAS HOUSTON

Institutional Review Board
Office of Research and Sponsored Programs
P.O. Box 425619, Denton, TX 76204-5619
940-898-3378 FAX 940-898-4416
e-mail: IRB@twu.edu

March 1, 2012

Ms. Kathleen Davis
2620 Picadilly Lane
Denton, TX 76209

Dear Ms. Davis:

Re: Correlation of Indicators of Adiposity with Advanced Glycation Endproducts (AGE), Soluble Receptor for AGE (sRAGE) and Other Inflammatory Markers (Protocol #: 16967)

The above referenced study has been reviewed by the TWU Institutional Review Board (IRB) and was determined to be exempt from further review.

If applicable, agency approval letters must be submitted to the IRB upon receipt PRIOR to any data collection at that agency. Because a signed consent form is not required for exempt studies, the filing of signatures of participants with the TWU IRB is not necessary.

Any modifications to this study must be submitted for review to the IRB using the Modification Request Form. Additionally, the IRB must be notified immediately of any unanticipated incidents. If you have any questions, please contact the TWU IRB.

Sincerely,

Kathy DeOrnellas, PhD.

Dr. Kathy DeOrnellas, Chair
Institutional Review Board - Denton

cc. Dr. Chandan Prasad, Department of Nutrition & Food Sciences
Dr. Victorine Imrhan, Department of Nutrition & Food Sciences
Graduate School

APPENDIX B

Institutional Review Board Approval, Modifications,
Extension and Consent Form Study II

TWU
TEXAS WOMAN'S UNIVERSITY
DENTON DALLAS HOUSTON

Institutional Review Board
Office of Research and Sponsored Programs
P.O. Box 425619, Denton, TX 76204-5619
940-898-3378 FAX 940-898-4416
e-mail: IRB@twu.edu

January 10, 2012

Ms. Kathleen Davis
2610 Picadilly Lane
Denton, TX 76209

Dear Ms. Davis:

Re: Effect of High Fat Versus Low Fat Diet on Advanced Glycation Endproducts, Receptor for Advanced Glycation End Products, and Inflammatory Cytokines in Healthy, Overweight or Obese Adults (Protocol #: 16870)

The above referenced study has been reviewed by the TWU Institutional Review Board (IRB) and appears to meet our requirements for the protection of individuals' rights.

If applicable, agency approval letters must be submitted to the IRB upon receipt PRIOR to any data collection at that agency. A copy of the approved consent form with the IRB approval stamp and a copy of the annual/final report are enclosed. Please use the consent form with the most recent approval date stamp when obtaining consent from your participants. The signed consent forms and final report must be filed with the Institutional Review Board at the completion of the study.

This approval is valid one year from December 2, 2011. Any modifications to this study must be submitted for review to the IRB using the Modification Request Form. Additionally, the IRB must be notified immediately of any unanticipated incidents. If you have any questions, please contact the TWU IRB.

Sincerely,

Kathy DeOrnellas PhD.

Dr. Kathy DeOrnellas, Chair
Institutional Review Board - Denton

enc.

cc. Dr. Chandan Prasad, Department of Nutrition & Food Sciences
Dr. Victorine Imrhan, Department of Nutrition & Food Sciences
Graduate School

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TWU INSTITUTIONAL REVIEW BOARD (IRB)

MODIFICATION REQUEST FORM

Complete this form when you would like to request a change on an approved study. This change could be a change in the research team, data collection sites, protocol (e.g., compensation, study procedures, etc.), and/or the informed consent. Submit this signed form along with copies of any new or modified materials you describe below to the IRB. NOTE: You may not implement any changes to an IRB-approved study until your Modification Request has been approved.

PRINCIPAL INVESTIGATOR: Kathleen Davis

DATE APPROVED BY IRB (most recent): 1/10/2012

TITLE OF STUDY: Effect of high fat versus low fat diet on advanced glycation endproducts, receptor for advanced glycation end products, and inflammatory cytokines in healthy, overweight or obese adults.

Provide a detailed description of the modification(s) requested:

We request permission to do a smaller, pilot study as a test run. This study will involve the same type of study participants as originally approved. The study intervention would change from one week of feeding for each phase of the project (there are currently two feeding phases/two diets) to one day of feeding for each phase of the study. The wash-out period would be reduced from four to two weeks, and consequently the benefits to participants would be reduced from $50 to $5 cash and a three hour weight management seminar in place of a day-long seminar. The blood draw schedule would change from just two draws on the morning of each study diet to one fasting draw, followed by breakfast, followed by a one, two and three hour post-meal draw. The following morning, participants would return for a single, fasting blood draw. The same procedure would apply to the second study diet, which would be fed 2 weeks later.

Provide a list of any new or modified documents materials and attach these items to this form:

Consent Form
Recruitment Flyer
Recruitment Script

Principal Investigator Assurance: I certify that the revised information provided for this project is correct and that no other procedures or forms will be used. I confirm that no changes will be implemented until I receive written approval for the changes from the TWU IRB.

Signature of Principal Investigator — Date: 1/26/12

APPROVED:

Signature of IRB Chair / Co-Chair — Date: 1/26/12

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TEXAS WOMAN'S UNIVERSITY

TWU INSTITUTIONAL REVIEW BOARD (IRB)

MODIFICATION REQUEST FORM

Complete this form when you would like to request a change on an approved study. This change could be a change in the research team, data collection sites, protocol (e.g., compensation, study procedures, etc.), and/or the informed consent. Submit this signed form along with copies of any new or modified materials you describe below to the IRB. NOTE: You may not implement any changes to an IRB-approved study until your Modification Request has been approved.

PRINCIPAL INVESTIGATOR: Kathleen Davis

DATE APPROVED BY IRB (most recent): 2/6/2012

TITLE OF STUDY: Effect of high fat versus low fat diet on advanced glycation endproducts, receptor for advanced glycation end products, and inflammatory cytokines in healthy, overweight or obese adults.

Provide a detailed description of the modification(s) requested:

(1) We request permission to expand recruitment to include electronic media including a portal announcement, Blackboard announcements, where permitted by the instructor, e-mail to doctor's offices and local offices such as libraries. We would also like to establish a Facebook account for the study. (2) The following exclusion criteria have been added to the consent form: "Participants must not be smokers; you must not have smoked or used smokeless tobacco products in the last 6 months. You must not be pregnant or lactating." (3) We have added questions on smoking, pregnancy, lactation, and race to the screening form. (4) In addition, we have updated the data collection form to reflect the new procedure for the blood draw for the pilot study. Each of the items which have been modified are attached with modified areas highlighted and numbered. The original of the consent form is also attached.

Provide a list of any new or modified documents materials and attach these items to this form:

Portal Announcement
Consent Form
Screening Questionnaire
Data Collection Form

Principal Investigator Assurance: I certify that the revised information provided for this project is correct and that no other procedures or forms will be used. I confirm that no changes will be implemented until I receive written approval for the changes from the TWU IRB.

Signature of Principal Investigator — Date: 2/7/12

APPROVED:

Signature of IRB Chair / Co-Chair — Date: 2/7/12

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RESEARCH & SPONSORED PROGRAMS
TEXAS WOMAN'S UNIVERSITY

MEMORANDUM

TO: Dr. Kathy DeOrnellas, Chair, Institutional Review BOARD
FROM: Kathleen Davis, Doctoral Candidate; Victorine Imrhan, Professor
RE: Effect of High Fat Versus Low Fat Diet on Advanced Glycation Endproducts, Receptor for Advanced Glycation End Products, and Inflammatory Cytokines in Healthy, Overweight or Obese Adults **ADDENDUM**
DATE: June 20, 2012

In order to better recruit participants for the above study, we would like to increase payment to participants from $5 to $50. This will be provided in place of the weight management course (approximate value $45) in order to provide equivalent benefits between the group of participants who have already completed the study (n=8) and those yet to complete it. This decision was based on feedback from potential recruits.

Approved
Rhonda Buckley
7/13/12

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TEXAS WOMAN'S UNIVERSITY

TWU INSTITUTIONAL REVIEW BOARD (IRB)
MODIFICATION REQUEST FORM

Complete this form when you would like to request a change on an approved study. This change could be a change in the research team, data collection sites, protocol (e.g., compensation, study procedures, etc.), and/or the informed consent. Submit this signed form along with copies of any new or modified materials you describe below to the IRB. NOTE: You may not implement any changes to an IRB-approved study until your Modification Request has been approved.

PRINCIPAL INVESTIGATOR: Kathleen Davis

DATE APPROVED BY IRB (most recent): ~~7/16/2012~~ 12/2/2012 (mod 7/13/12) fcl

TITLE OF STUDY: Effect of high fat versus low fat diet on advanced glycation endproducts, receptor for advanced glycation end products, and inflammatory cytokines in healthy, overweight or obese adults.

Provide a detailed description of the modification(s) requested:

(1) We request permission to add Stacy Gage to the list of team members. A copy of her NIH training certificate is attached. (2) We also request permission to extend the proposed analysis of the study to use data collected from the 5 day food record to compare nutrients consumed as well as dietary AGE consumed to serum AGE, sRAGE, and other biomarkers already approved to be analyzed for the study. (3) Finally, small revisions in wording of the Portal Announcement, and Telephone and E-mail Recruiting Script have been made to reflect the changes in compensation for the study, the need for participants to be non-smokers, and the changes in dates for the study, now extended to fall 2012 and spring 2013.

Provide a list of any new or modified documents materials and attach these items to this form:

Portal Announcement
Telephone and E-mail Recruiting Script

Principal Investigator Assurance: I certify that the revised information provided for this project is correct and that no other procedures or forms will be used. I confirm that no changes will be implemented until I receive written approval for the changes from the TWU IRB.

Signature of Principal Investigator — Date: 11/14/2012

APPROVED:

Signature of IRB Chair / Co-Chair — Date: 11/20/12

TWU
TEXAS WOMAN'S UNIVERSITY
DENTON DALLAS HOUSTON

Institutional Review Board
Office of Research and Sponsored Programs
P.O. Box 425619, Denton, TX 76204-5619
940-898-3378 FAX 940-898-4416
e-mail: IRB@twu.edu

December 7, 2012

Ms. Kathleen Davis
2610 Picadilly Lane
Denton, TX 76209

Dear Ms. Davis:

Re: Effect of High Fat Versus Low Fat Diet on Advanced Glycation Endproducts, Receptor for Advanced Glycation End Products, and Inflammatory Cytokines in Healthy, Overweight or Obese Adults (Protocol #: 16870)

The request for an extension of your IRB approval for the above referenced study has been reviewed by the TWU Institutional Review Board (IRB) and appears to meet our requirements for the protection of individuals' rights.

If applicable, agency approval letters must be submitted to the IRB upon receipt PRIOR to any data collection at that agency. A copy of the approved consent form with the IRB approval stamp is enclosed. Please use the consent form with the most recent approval date stamp when obtaining consent from your participants. A copy of the signed consent forms must be submitted with the request to close the study file at the completion of the study.

This extension is valid one year from December 2, 2012. Any modifications to this study must be submitted for review to the IRB using the Modification Request Form. Additionally, the IRB must be notified immediately of any unanticipated incidents. If you have any questions, please contact the TWU IRB.

Sincerely,

Rhonda R Buckley

Dr. Rhonda Buckley, Chair
Institutional Review Board - Denton

cc. Ronald Hovis, Department of Nutrition & Food Sciences
Dr. Victorine Imrhan, Department of Nutrition & Food Sciences
Graduate School

TEXAS WOMAN'S UNIVERSITY
CONSENT TO PARTICIPATE IN RESEARCH

Title: Effect of high fat versus low fat diet on advanced glycation endproducts, receptor for advanced glycation end products, and inflammatory cytokines in healthy, overweight or obese adults.

Investigator: Kathleen Davis ..... KDavis10@twu.edu 940/898-2639
Advisor: Vickie Imrhan, PhD ..... VImrhan@twu.edu 940/898-2650

Explanation and Purpose of the Research

You are being asked to participate in a research study in the Department of Nutrition and Food Sciences at Texas Woman's University. The purpose of the research is to study the effects of eating both a high fat and a low fat diet rich in advanced glycation end products (AGEs)—which occur when high heat is used in cooking like in baking, grilling or frying—on inflammatory markers in the blood. Markers we will measure are: interleukin-6, C-reactive protein and interleukin-10. We will also look at the effects of the two diets on blood levels of AGE and a receptor for AGE (sRAGE).

You are being asked to participate because you are between the ages of 20-45, have a BMI of 27-35, and you have said that you do not have high blood pressure, heart disease, diabetes, kidney disease, metabolic disease or cancer. Participants must not be smokers; you must not have smoked or used smokeless tobacco products in the last 6 months. You must not be pregnant or lactating.

Description of Procedures

The study will require that you come to campus (Old Main Building) for a fasting blood draw on a Friday, stay for breakfast and stay for three (3) post meal blood draws, one, two, and three hours later. You will then return to campus (the Human Development Building) the same day for lunch, dinner and a snack and will come back to campus the next day (Saturday) for another fasting blood draw. You will be allowed to eat only the study foods during that day. Please do not eat other foods during the study period. During the first visit to campus, a researcher of the same sex will weigh you, measure your height and measure your waist in a screened area. A certified phlebotomist (person taking your blood) or nurse will then draw about 12 tablespoons of your blood.

Two weeks later we will repeat the same schedule. You will come to campus (Old Main Building) for a fasting blood draw on a Friday, stay for breakfast and stay for three (3) post meal blood draws, one, two, and three hours later. You will then return to campus (the Human Development Building) the same day for lunch, dinner and a snack and will come back to campus the next day (Saturday) for another fasting blood draw. You will be allowed to eat only the study foods during that day. Please do not eat other foods during the study period.

This study will also require that you keep one five day food diary prior to the study. This study will require about 13 hours of time from you during a four week period.

You will receive a diet that is high fat (40 percent fat) during one study day and a diet that is low fat (20% fat) another study day. Each diet will have the same number of calories. Both diets will be high in AGEs and will be cooked using high heat cooking methods such as broiling, roasting, baking, grilling, and frying. You may eat ONLY the study foods during each one day intervention. Let us know if you are still hungry after eating the study foods so that we can increase your portions.

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Time Commitment

The study will take about 4 weeks to complete. Prior to the study you will need to complete the screening and informed consent (1 hour). You will also keep a five day food record before beginning the study (2 hours).

During week one, you will come to the lab to have your height, weight, and waist measured, have a fasting blood draw, a breakfast provided by us, and a 1, 2, and 3 hour post meal blood draw (3.5 hours). You will also eat lunch and dinner in the lab that day (1 hour).The next day you will report to the lab for another fasting blood draw (30 minutes).

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Total		**13 hours**

Total time commitment: 13 hours over a 4 week time period.

Potential Risks

Possible risks related to participating in the study include loss of confidentiality, emotional discomfort and embarrassment, physical discomfort relating to the blood draw, infection and bleeding due to the blood draw, allergic reactions or food allergies, loss of time, and fatigue.

To protect your confidentiality, you will be given a 3 digit code, which will be used on all records. Only the researchers will know the identity of participants. All records will be stored in Dr. Imrhan's office, which is kept locked. Paper data will be shredded within 5 years of completion of the study and computer data will be deleted permanently from programs and hard drives. Blood will be labeled only with an ID number and will not be able to be identified as yours. No names or other identifying information will be included in any publication relating to the study. Confidentiality will be protected to the extent that is allowed by law. There is a possible loss of confidentiality in all e-mail, down-loading, and Internet transactions.

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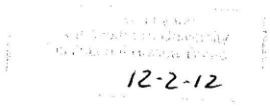

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Another possible risk is loss of time and fatigue. You were told about the time involved in this study which is about 34 hours over 6 weeks. To lessen boredom during the longer visits to Old Main, you may bring homework or other work to do in between working with the researchers. We will also provide a DVD for you to watch if you prefer.

The researchers will try to prevent any problem that could happen because of this research. You should let the researchers know at once if there is a problem, and they will help you. However, TWU does not provide medical services or financial assistance for injuries that may happen because you are taking part in this research.

Participation and Benefits

Your involvement in this study is voluntary, and you may stop participating at any time without penalty. As a participant you will receive breakfast, lunch and dinner for two days. You will receive the results of your blood collection within 6 months of participating in the research. You will also receive $50.

Questions Regarding the Study

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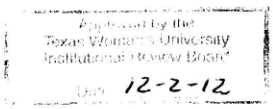
Signature of Participant **Date**



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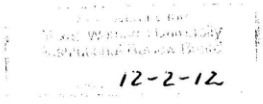

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Signature of Participant **Date**

Approved by the
Texas Woman's University
Institutional Review Board
Date: 12-2-11

Revised: 7-13-12

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E-mail: _____

or

Address:

_____

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APPENDIX C

Biochemical Analysis Procedures

Glucose Procedure:

Thaw Samples:

1. Begin thawing samples.
2. Turn on water bath.

Aliquot into Smaller Samples:

3. Aliquot 200 µl from thawed samples into microcentrifuge tubes.
4. Set up template for Wells Plate.
5. Label test tubes.

Prepare standards according to the following dilution:

1. S25: 12.5 µl standard; 37.5 µl DI water
2. S50: 25 µl standard; 25 µl DI water
3. S100: 50 µl standard

Transfer to Test Tubes:

1. Pipette 1 ml of reagent into each test tube.
2. Add 10 µl of DI water to blanks.

Note time:

3. Add 10 µl of standard to appropriate tubes
4. Add 10 µl of sample to appropriate tubes.
5. Vortex each test tube.

Incubate:

Put test tube racks in water bath at 37 degrees C for 5 minutes

Transfer to Wells Plate:

Begin transferring 300 µl from each test tube to the appropriate well on the Wells Plate.

Note time: Read plate by 60 minutes from the time you begin putting samples and standards into test tubes. Read at 500 nm.

Triglyceride Procedure:

Thaw Samples:

1. Begin thawing samples.
2. Turn on water bath.

Aliquot into Smaller Samples:

1. Aliquot 200 µl from thawed samples into microcentrifuge tubes.
2. Set up template for Wells Plate.
3. Label test tubes.

Prepare standards according to the following dilution:

1. S50: 12.5 µl standard; 37.5 µl DI water
2. S100: 25 µl standard; 25 µl DI water
3. S200: 50 µl standard

Prepare activated reagent:

1. Add 50 µl of activator to every 5 ml of regent. (i.e. For 150 ml of reagent, add 1.5 ml of activator).

Transfer to Test Tubes:

1. Pipette 1 ml of activated reagent into each test tube.
2. Add 10 µl of DI water to blanks.

Note time:

1. Add 10 µl of standard to appropriate tubes
2. Add 10 µl of sample to appropriate tubes.
3. Vortex each test tube.

Incubate:

Put test tube racks in water bath at 37 degrees C for 5 minutes

Transfer to Wells Plate:

Begin transferring 300ml from each test tube to the appropriate well on the Wells Plate.

Note time: Read plate by 60 minutes from the time you begin putting samples and standards into test tubes. Read at 500 nm.

Cholesterol Procedure:

Thaw Samples:

1. Begin thawing samples.
2. Turn on water bath.

Aliquot into Smaller Samples:

1. Aliquot 200 µl from thawed samples into microcentrifuge tubes.
2. Set up template for Wells Plate.
3. Label test tubes.

Prepare standards according to the following dilution:

1. S50: 12.5 µl standard; 37.5 µl DI water
2. S100: 25 µl standard; 25 µl DI water
3. S200: 50 µl standard

Transfer to Test Tubes:

1. Pipette 1 ml of reagent into each test tube.
2. Add 10 µl of DI water to blanks.
3. ***Note time:***
4. Add 10 µl of standard to appropriate tubes
5. Add 10 µl of sample to appropriate tubes.
6. Vortex each test tube.

Incubate:

Put test tube racks in water bath at 37 degrees C for 5 minutes

Transfer to Wells Plate:

Begin transferring 300 µl from each test tube to the appropriate well on the Wells Plate.

Note time: Read plate by 60 minutes from the time you begin putting samples and standards into test tubes. Read at 500 nm.

HDL Cholesterol Procedure:

Thaw Samples:

1. Begin thawing samples.
2. Turn on water bath.

HDL Separation:

1. Using a microcentrifuge tube, pipette 200 µl of serum or plasma into the tube.
2. Add 20 µl of HDL precipitating reagent
3. Vortex well and allow to stand 5 minutes
4. Centrifuge for 10 minutes at high speed (10 x g).

Prepare standards according to the following dilution:

S25: 50 µl standard; 50 µl DI water
S50: 50 µl standard
S100: 25 µl of TChol standard, 25 µl of DI water

Transfer to Test Tubes:

1. Pipette 1 ml of reagent into each test tube.
2. Add 25 µl of DI water to blanks.

Note time:

3. Add 25 µl of standard to appropriate tubes
4. Add 25 µl of clear supernatant from each sample to appropriate tubes.
5. Vortex each test tube.

Incubate:

Put test tube racks in water bath at 37 degrees C for 5 minutes

Transfer to Wells Plate:

1. Begin transferring 300 µl from each test tube to the appropriate well on the Wells Plate.

Note time: Read plate by 60 minutes from the time you begin putting samples and standards into test tubes. Read at 500 nm.

HMW Adiponectin Procedure: (Alpco)

Reagents:
Bring reagents to room temperature.

Protease II: (stable for 2 days at 2-10 degrees C; stable for the life of the kit @ -20C)
Reconstitute Protease II:

- Add 10 ml of Protease buffer to the Protease II vial.
- Dissolve completely at room temperature for 15-30 minutes.

Samples: (stable at 2-10 degrees C for 2 days)

- Bring to room temperature.
- Aliquot samples.
- Label microcentrifuge tubes for pretreated samples AND for diluted samples.
- Add 100 mcl of Protease II to 10 mcl of each sample.
- Incubate at 37 degrees C for 20 minutes.
- Immediately add 400 mcl of sample pretreatment buffer.
- Vortex.
- Dilute pretreated samples: add 10 mcl of the pretreated sample to 1 ml of Dilution Buffer.
- Vortex. **(DILUTED SAMPLES MUST BE USED WITHIN 2 HRS OF DILUTION.)**

Wash Buffer: (Stable at 2-10 degrees C for life of kit)

- Dilute wash buffer concentrate with 900 ml of distilled water.

Low, High and Serum Controls: (can be stored at -20 degrees C)
Reconstitute Controls:

- Add 1 ml DI water to each vial.
- Replace the rubber stopper and cap.
- Gently swirl each vial and let stand for 10 minutes prior to use.
- The contents should be in solution with NO VISIBLE PARTICULATE matter.

Standards:

- **Make 8 standards JUST PRIOR TO LOADING PLATE**

Standard #	Calibrator	Dilution Buffer	Final Concn
1	10 mcl	1000 mcl	4.8 ng/ml
2	150 mcl Std1	150	2.4
3	150 mcl Std2	150	1.2
4	150 mcl Std3	150	0.6
5	150 mcl Std4	150	0.3
6	150 mcl Std5	150	0.15
7	150 mcl Std 6	150	0.075
8	0	150 mcl	0

Substrate: (Use immediately after reconstitution and waste any excess.)

Just prior to use: add 6 ml Substrate Buffer to the Substrate vial.

Assay:

First Incubation:

1. Label/plan your adiponectin template.
2. Add 50 **mcl of each standard (8 PLUS HC, LC AND SC) and each sample** into the appropriate wells.
3. Cover and incubate at room temperature for 1 hour.

First Wash

1. Decant the plate. Strike against towels.
2. Wash with 350-400 mcl WB per well using squeeze bottle or automated plate washer.
3. Decant the plate. Strike against towels.
4. Wash two more times (3 washes).

Second Incubation:

1. Add 50 mcl of biotin labeled monoclonal antibody to each well.
2. Cover with a plate sealer and incubate for 1 hour at room temperature.

Second Wash:

Repeat first wash procedure.

Third Incubation:

1. Add 50 mcl of enzyme labeled streptavidin.
2. Cover with a plate sealer and incubate for 30 minutes at room temperature.

Third Wash:

Repeat first wash procedure:

Fourth Incubation:

1. Add 50 mcl substrate solution to each well.
2. Protect the plate from light and incubate for 10 minutes at room temperature.

Stop:

1. Add 50 mcl stop reagent to each well.
2. Read at 492 nm 10-30 minutes after the addition of the stop reagent.

CML ELISA Assay

Prepare Working Solutions:

Wash buffer:

- Add 20 ml wash buffer (10x) to 180 ml DI water.
- Store at 2-8 degrees for one day only

Guidelines:

1. Bring all working solutions to 15-25 degrees C.
2. Do NOT aliquot standard, antibodies or conjugate.
3. Centrifuge standard and conjugate briefly at 6000 rpm x 2 minutes before use. Mix carefully. Do NOT vortex. Take only the clear supernatant.
4. Use plastics, not glassware.

Pre-treat samples:

1. Incubate in a 0.5 ml microcentrifuge tube: (22 x dilution)
    - 100 µl wash buffer
    - 5 µl sample serum
    - 5 µl proteinase K.

2. Incubate for 2 hours at 37 degrees C (waterbath).

3. Inactivate proteinase K at 80 degrees C for 10 minutes (time starts when 80 degrees reached) using thermoblock.

4. Cool sample to room temp.

Begin Assay:

Add Bi-BSA-AGE: (have 250 µl of BSA-AGE)

1. Add 70 µl Bi-BSA-AGE to 14 ml assay buffer solution.
2. Pipet 100 µl Bi-BSA-AGE solution in all wells (except A1 and A2).
3. Cover with the adhesive foil cover.
4. Incubate for 1 hour at constant shaking at 15-25 degrees C.

Wash:

1. Wash 3 times with 300 µl wash buffer.

2. Remove buffer by aspirating or tapping out solution, especially at last step.

Add samples and standards: (have 40 µl conjugate)

1. Add 10 µl MAB-CML-HRP conjugate to 14.5 ml assay buffer.
2. Add 50 µl standards, positive controls and diluted samples to wells.
3. Immediately add 50 µl MAB-CML-HRP conjugate in wells except A1 and A2
4. Cover with an adhesive foil.
5. Incubate for 1 hour with constant shaking at 15-25 degrees C.

Wash:

1. Wash 3 times with 300 µl wash buffer.
2. Remove buffer by aspirating or tapping out solution, especially at last step.

Add ABTS solution:

1. Add 100 µl ABTS solution to all wells.
2. Cover with an adhesive foil.
3. Incubate for 30 minutes with constant shaking at 600 rpm at 15-25 degrees C.

Measure:

1. Measure the OD of each well at 405 nm, reference 492 nm.
2. If reading for standard 0 ng/ml is <1 E, prolong incubation to 45-60 minutes.
3. Use 4 parameter fit method to calculate curve.

Biovendor sRAGE ELISA Assay

Prepare Working Solutions:

Bring all reagents to room temp.

Wash buffer:

- Add 100 ml wash buffer (10x) to 900 ml DI water.
- Store at 2-8 degrees for up to one month.

Guidelines:

1. Bring all working solutions to 15-25 degrees C.
2. Use standard immediately or store at -20 degrees C for up to 3 months. Avoid freezing and thawing repeatedly. Do NOT store diluted standard solutions.
3. Use plastics or very clean glassware.

Samples:
1. Dilute samples 3x with dilution buffer just prior to assay: 100 µl of sample per 200 µl dilution buffer. Vortex to mix, but do not allow to foam. Do not store diluted samples. Samples should be thawed just prior to assay. Avoid repeated freezing and thawing. Avoid using hemolyzed or lipemic samples.

Standards and Controls:

- Reconstitute the lyophilized master standard with dilution buffer just before assaying. Let it dissolve for 15 minutes with occasional gentle shaking (do not allow to foam). The stock solution is 3200 pg/ml.
- Prepare standards:

Volume of Standard	**Dilution Buffer**	**Concentration**
Stock	-	3200 pg/ml
250 µl of stock	250 µl	1600 pg/ml
250 µl of 1600 pg/ml	250 µl	800 pg/ml
250 µl of 800 pg/ml	250 µl	400 pg/ml
250 µl of 400 pg/ml	250 µl	200 pg/ml
250 µl of 200 pg/ml	250 µl	100 pg/ml
250 µl of 100 pg/ml	250 µl	50 pg/ml

- Refer to the CofA for the current volume of dilution buffer needed to reconstitute the quality controls.

- Reconstitute each control just prior to assay. Allow to dissolve for 15 minutes with occasional gentle shaking (not to foam). Use them AS IS. DO NOT DILUTE.

Begin Assay:

5. Add 100 µl of Standards, Quality Controls, Dilution Buffer (blank) and diluted samples in duplicate to each well.
6. Cover and incubate at room temp (25 degrees C) x 2 hours at 300 rpm on orbital plate shaker.
7. Wash x5 with wash solution (350 µl per well) After final wash, invert and tap plate strongly against a paper towel.
8. Add 100 µl of biotin labeled antibody to each well.
9. Incubate at room temp (25 degrees C) x 1 hour at 300 rpm on orbital plate shaker.
10. Wash x5 with wash solution (350 µl per well) After final wash, invert and tap plate strongly against a paper towel.
11. Add 100 µl of streptavidin HRP conjugate into each well.
12. Incubate at room temp (25 degrees C) x 30 minutes at 300 rpm on orbital plate shaker.
13. Wash x5 with wash solution (350 µl per well) After final wash, invert and tap plate strongly against a paper towel.
14. Add 100 µl of substrate solution into each well. Avoid exposing to sunlight. Cover the plate with foil.
15. Incubate the plate x 10 minutes at room temperature. The incubation can be extended up to 20 if the temp is below 20 degrees. Do not shake during incubation.
16. Stop the color development by adding 100 µl of stop solution.
17. Determine the absorbance by measuring at 450 nm with reference wavelength set to 630 nm.

APPENDIX D

Recruiting Flyers and Announcements

WANTED: Participants

Are you . . . ?

- Age 20-45?
- Overweight?
- Healthy?

We need men and women to participate in a study about the effect of diet on AGE (advanced glycation end products) and inflammation.

You will receive:

- Free food x 2 days.
- $5 compensation at the end of the study.
- **A FREE three-hour seminar on weight management.**

(You must be ages 20-45, BMI 27-35, and free of high blood pressure, diabetes, heart disease, cancer, & kidney disease):

To find out if you qualify to participate:

Call or e-mail Kathleen Davis:

KDavis10@twu.edu; 940-395-8577

This is for research purposes only and is VOLUNTARY.

There is a potential risk of loss of confidentiality in all email, downloading, and Internet transactions

Portal Announcement/Facebook Announcement:

WANTED: Participants!! Are you . . . ?

- Age 20-45?
- Overweight?
- Healthy?

We need men and women to participate in a study about the effect of diet on AGE (advanced glycation end products) and inflammation. You will receive: free food x 2 days, $5 compensation at the end of the study, and **A FREE three-hour seminar on weight management.** *(You must be ages 20-45, BMI 27-35, and free of high blood pressure, diabetes, heart disease, cancer, & kidney disease):*

To find out if you qualify to participate: Call or e-mail Kathleen Davis: KDavis10@twu.edu; 940-395-8577. ***This is for research purposes only and is VOLUNTARY.*** *There is a potential risk of loss of confidentiality in all email, downloading, and Internet transactions*

APPENDIX E

Recruitment Script

Telephone Recruitment Script for Potential Participants in AGE Feeding Study

Researcher:

Thank you for contacting me regarding participating in our study at TWU Nutrition and Food Sciences on foods containing advanced glycation end products.

We are looking for participants who are 20-45 years old, both male and female, who are overweight and willing to eat our study foods for two days during this spring (2012). If you would like, I will send you a chart to help you tell if you meet our study criteria. May I please have your e-mail address?

I am sending you a chart to use to determine your BMI. We need people whose BMI's are between 27 and 35. If you would like to participate, send me a note back, and I will schedule a time to meet with you to fill out your consent form.

Thank you again!

E-mail Script:

Thank you for contacting me regarding participating in our study at TWU Nutrition and Food Sciences on foods containing advanced glycation end products. We are looking for participants who are 20-45 years old, both male and female, who are overweight and willing to eat our study foods for two days during this spring (2012).

Use This Chart to Determine Your BMI: (We are looking for people with a BMI between 27 and 35.)

BMI	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**31**	**32**	**33**	**34**	**35**
Ht	**Body Weight (pounds)**																
58 (4'10")	91	96	100	105	110	115	119	124	129	134	138	143	148	153	158	162	167
59 (4'9")	94	99	104	109	114	119	124	128	133	138	143	148	153	158	163	168	173
60 (5')	97	102	107	112	118	123	128	133	138	143	148	153	158	163	168	174	179
61 (5'1")	100	106	111	116	122	127	132	137	143	148	153	158	164	169	174	180	185
62 (5'2")	104	109	115	120	126	131	136	142	147	153	158	164	169	175	180	186	191
63 (5'3")	107	113	118	124	130	135	141	146	152	158	163	169	175	180	186	191	197
64 (5'4	110	116	122	128	134	140	145	151	157	163	169	174	180	186	192	197	204

BMI	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**31**	**32**	**33**	**34**	**35**
Ht	**Body Weight (pounds)**																
")																	
65 (5'5")	114	120	126	132	138	144	150	156	162	168	174	180	186	192	198	204	210
66 (5'6")	118	124	130	136	142	148	155	161	167	173	179	186	192	198	204	210	216
67 (5'7")	121	127	134	140	146	153	159	166	172	178	185	191	198	204	211	217	223
68 (5'8")	125	131	138	144	151	158	164	171	177	184	190	197	203	210	216	223	230
69 (5'9")	128	135	142	149	155	162	169	176	182	189	196	203	209	216	223	230	236
70 (5'10)	132	139	146	153	160	167	174	181	188	195	202	209	216	222	229	236	243
71 (5'11")	136	143	150	157	165	172	179	186	193	200	208	215	222	229	236	243	250
72 (6')	140	147	154	162	169	177	184	191	199	206	213	221	228	235	242	250	258

BMI	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**31**	**32**	**33**	**34**	**35**
Ht	**Body Weight (pounds)**																
73 (6’1”)	144	151	159	166	174	182	189	197	204	212	219	227	235	242	250	257	265
74 (6’2”)	148	155	163	171	179	186	194	202	210	218	225	233	241	249	256	264	272
75 (6’3”)	152	160	168	176	184	192	200	208	216	224	232	240	248	256	264	272	279
76 (6’4”)	156	164	172	180	189	197	205	213	221	230	238	246	254	263	271	279	287

Source: http://www.nhlbi.nih.gov/guidelines/obesity/bmi_tbl.htm

The study will require that you come to campus (Old Main Building) for a fasting blood draw on a Friday, stay for breakfast and stay for three (3) post meal blood draws, one, two, and three hours later. You will then return to campus (the Human Development Building) the same day for lunch, dinner and a snack and will come back to campus the next day (Saturday) for another fasting blood draw. You will be allowed to eat only the study foods during that day. Please do not eat other foods during the study period. During the first visit to campus, a researcher of the same sex will weigh you, measure your height and measure your waist in a screened area. A certified phlebotomist (person taking your blood) or nurse will then draw about 12 tablespoons of your blood.

Two weeks later we will repeat the same schedule. You will come to campus (Old Main Building) for a fasting blood draw on a Friday, stay for breakfast and stay for three (3) post meal blood draws, one, two, and three hours later. You will then return to campus (the Human Development Building) the same day for lunch, dinner and a snack and will come back to campus the next day (Saturday) for another fasting blood draw. You will be allowed to eat only the study foods during that day. Please do not eat other foods during the study period.

This study will also require that you keep one five day food diary prior to the study. This study will require about 13 hours of time from you during a four week period.

You will receive a diet that is high fat (40 percent fat) during one study day and a diet that is low fat (20% fat) another study day. Each diet will have the same number of calories. Both diets will be high in AGEs and will be cooked using high heat cooking methods such as broiling, roasting, baking, grilling, and frying. You may eat ONLY the study foods during each one day intervention. Let us know if you are still hungry after eating the study foods so that we can increase your portions.

To thank you for your participation, you will receive $5 compensation, and you will be invited to participate in a free three-hour seminar on weight management. If you wish to receive the newsletter, you will need to provide your mailing or e-mail address. If you would like to receive your blood results from the study, you will need to provide your mailing or e-mail address.

If you would like to participate in the study, please call or e-mail me: 940-395-8577 or KDavis10@twu.edu. I will set a time for you to come to campus to meet with me and sign a consent form. Then we will be able to answer further questions that you may have.

Thank you for your time. Please call me (940-395-8577) or respond to this e-mail (KDavis10@twu.edu), and we will set up a time to meet with you.

APPENDIX F

Screening Questionnaire

SCREENING QUESTIONNAIRE

ID: _____

Consent Obtained:_____

Sex: F_____ M_____ Age_____

Telephone:_____Email: _____

Do you smoke? Y_____ N_____ Have you smoked or used smokeless tobacco in the past 6 months? ____

Are you pregnant? Y___N_____ Are you lactating?Y___N_____

Weight_____ Height_____ BMI_____

Medical condition(s) for which you are taking medicine:

Hypertension_____High Cholesterol_____ Kidney Disease_____Diabetes_____

Lung Disease _____Heart Disease_____Liver Disease_____

Other Conditions For Which you Take Medication_____

Has a doctor ever told you that you have any of the following diseases?

High blood pressure_____ High cholesterol_____Heart disease_____

Diabetes_____ Kidney Disease_____ Metabolic Disease_____

Cancer_____

_____

List any medications, drugs, prescription drugs, over the counter drugs, vitamins or food supplements you are taking: List amount (mg) and times taken (daily, weekly, etc.)

Name	**Amount (mg)**	**Times Taken (daily, weekly, etc.)**

Do you have any food allergies? _____N _____Y What are they?_____

_____

Which race/ethnicity do you identify with? (you may select more than one)

_____White/Caucasian

_____Black/African-American

_____Asian

_____Hispanic

_____Native American

_____Other

APPENDIX G

Food Journal Form

Food Journal

ID:

Date & time	Food, drink, snack, how prepared and amount. Example: chicken breast, breaded, fried in oil	Location (ex Home, car, bar restaurant)	Do not write in this column

APPENDIX H

Data Collection Form

Data Collection Form

Participant #_____

Date_____(initial draw)

Diet_____

BMI	Weight	Height	Waist Circum.

Action	Time
Blood Drawn Fasting	
Consumed Breakfast	
Blood Drawn 1 Hours after eating	
Blood Drawn 2 Hours after eating	
Blood Drawn 3 Hours after eating	

Date_____(Follow-Up Draw)

Diet_____

Action	Time
Blood Drawn Fasting	

APPENDIX I

Sample Menus

Sample Menus

Meal	Low-Fat Diet	High Fat Diet
Breakfast	30 g Rice Krispies cereal [600] 250 ml Fat free milk [4] 25 g (1 slice) whole wheat toast [21] w/2.5 g Smart Balance [156] 90 g smoked deli ham [2114] 85 g banana [8] 120 ml 100% apple juice [3] Total AGE Meal: [2906]	30 g Life cereal [394] 250 ml Whole milk [12] 25 g (1 slice) whole wheat toast [21] w/5 g Smart Balance [311] Bacon, microwaved (20 g) [1805] 85 g banana [8] 120 ml 100% apple juice [3] Total AGE Meal: [2554]
Lunch	Toasted cheese sandwich: 25 g x 2 whole wheat bread [42] 30 g reduced fat cheddar cheese [737] 2.5 g Smart Balance [156] 50 g carrot sticks [5] 250 ml chicken noodle soup [4] 100 g baked apples with 15 ml honey [46] 250 ml Coke [7] Total AGE Meal: [997]	Toasted cheese sandwich: 25 g x 2 whole wheat bread [42] 30 g regular cheddar cheese [1657] 5 g Smart Balance [311] 50 g carrot sticks [5] 250 ml chicken noodle soup [4] 100 g apples-no skin [13] 250 ml diet Coke [3] Total AGE Meal: [2035]
Dinner	90 g broiled chicken breast [5245] 100 g Grilled California veggies (broccoli, carrots, cauliflower) [226] (1.25 tsp corn oil) [150] 100g roasted potatoes [218] 35 g whole grain roll [29] 2.5gl Smart Balance [156] 60 g Frozen fruit bar [11] 120 ml orange juice [7] 360 ml Diet Coke (12 oz can) [4] Total AGE Meal: [6046]	90 g pan fried chicken breast [4444] 100 g Grilled California veggies (broccoli, carrots, cauliflower) [226] (1.25 tsp corn oil) [150] 50 g fried potatoes [347] 35 g whole grain roll [29] w/5 g Smart Balance [311] 60 g frozen fruit bar [11] 360 ml Diet Coke (12 oz can) [4] Total AGE Meal: [5522]
Snack 1	30 g Roasted cashews (1 oz) [2942] 250 ml fat-free milk [4] With 1 Tbsp syrup (chocolate or strawberry) Total AGE Snack: [2946]	30 g Roasted Almonds (1 oz) [1995] 250 ml whole milk [12] Total AGE Snack: [2007]
Snack 2	20 g cocktail peanuts [1667] 30 g Snyder's pretzel minis [537] 120 ml apple juice [3] Total AGE Snack: [2207]	28 g cocktail peanuts [2333] 30 g goldfish [653] Total AGE Snack: [2986]
Total AGE (kU)	2906+997+6046+2946+2207=15,102 KU (1.51×10^7)	2554+2035+5522+2007+2985=15,098 KU (1.51×10^7)

Total Calories	2249	2246
% Fat	20 % (50g) (0.7:1:0.6 SF:MUFA:PUFA)	41 % (104g) (0.9:1:0.5 SF:MUFA:PUFA)
Protein	108 g (19%)	103 g (18%)
Carb	356 g (62%)	235 g (41%)

APPENDIX J

Macronutrient Analysis by Independent Lab

POPE TESTING LABORATORIES, INC.
CONSULTING ANALYTICAL CHEMISTS
Food, Nutritional, Water and Agricultural Analysis
2220 Hinton Drive
Phone: 972-871-2892
Irving, TX 75061
972-871-7188
pope.labs@yahoo.com
Fax: 972-871-7452

March 8, 2012

Texas Woman's University
P.O. Box 425888
Denton, TX 76204

Attn: Nutrition & Food Science

Report of Tests on: Food

	Study Diet A	Study Diet B
Moisture	84.73 %	87.89 %
Oil (AH)	1.75 %	2.32 %
Ash	0.68 %	0.50 %
Nitrogen	0.48 %	0.31 %
Nitrogen (re-run)	0.52 %	0.48 %
Carbohydrates	9.84	7.37
Carbohydrates (w/ Nitrogen re-run)	9.59	6.32
Calories	67.11	58.04

Calculated Macronutrient Data Compared to Measured Data

	Diet A (LF HA): 3.07 kg		Diet B (HF HA): 3.37 kg	
Category	Pope	Calculated	Pope	Calculated
Fat	53.7 g (24%)	50 g (20%)	78.2 g (36%)	104 g (41%)
Protein	0.52% N 99.7 g (20%)	108 (19%)	0.48% N 101.1 g (21%)	103 g (18%)
Carbohydrate	302g (60%)	356 g (62%)	213 g (44 %)	235 g (41%)
Calories	2018 kcals	2249 kcals	1956 kcals	2246 kcals

APPENDIX K

CML Measurement in Food Procedure

CML Measurement in Food

- Prepare study meals.
- Homogenize the food at room temperature as needed using water to achieve homogenization.
- Thaw 80 g sample of each diet.
- Modification: sample was lyophilized, reconstituted to original concentration)
- Mix 1 ml of each sample with 9 ml 1x PBS. (10 x dilution).

Prepare three sets of samples for each diet (one untreated, one treated with Proteinase K, one treated with acid).

Proteinase K Treatment:

1. Need to know protein concentration of samples: in this case, it is approximately 30 mg/ml of each sample.
2. Apply Proteinase K (15.6 mg/ml) to food samples at a concentration of 0.2% (w/w). Weight/weight % = Grams solute/grams solution x 100.
    a. 0.2% = 0.2 mg Prot K protein/100 mg food protein
    = Food homogenate (non-dilute) contains ~100 g protein/3070 ml or about 30 mg/ml.
    10x dilute homogenate contains ~100 g protein/30.7 ml or about 3 mg/ml
    For 100 mg food protein, you would need 33.3 ml of non-dilute homogenate. (100/3)
    To provide 0.2 mg of protein from Prot K, you need 12.8 µl of proteinase K (15.6/1000=0.2/x)

    b. Thus, to provide a 0.2% w/w solution, you need 12.8 µl of prot K, and 33.3 ml – 12.8 or 33.287 ml of dilute food homogenate.

    c. This needs to be scaled down because proteinase K supply is very limited and only 50 µl of sample is needed per well. Instead we will use 2 µl Proteinase K/5.19 ml dilute food homogenate. (.0128/33.2 = .002/x)

    (To summarize: 2 µl proteinase K = 31.2 µg protein (0.312 mg) . 5.19 ml dilute food homogenate = 16 mg protein; .312/16 = 2% solution.

    Note: This is the concentration recommended by Dr. Uribarri. In the CML ELISA assay by Microcoat, the amount of proteinase K/serum protein is actually 22% w/w. If these concentrations were to be used, then you would need 1410 µl of prot K per 33.3 ml dilute homogenate, which would amount to 5 µl proteinase K per 118 µl dilute food homogenate.

3. Apply 2 µl proteinase K to 5.19 ml food homogenate.
4. Incubate at 37 degrees C overnight in the water bath.
5. Deactivate proteinase K at 65 degrees C for 1 hour.
6. Spin at 1000 rpm x 10 minutes.
7. Remove 50 µl of supernatant to use on CML assay.

Acid treatment:

1. Take 10x dilute homogenate.
2. Treat with 6 mm HCl. $C_1V_1=C_2V_2$ (Start with 8N HCl)
   $8*V_1=6^{-3}*2$
   $V_1=0.0015$
3. Add 1.5 µl 8 N HCl to 2 ml 10x dilute food homogenate.
4. Vortex. Allow to stand x 1 hour on orbital mixer.
5. Neutralize drop by drop using 5 M NaOH
6. When pH = 7.0, stop.
7. Centrifuge sample.
8. Apply 50 µl of supernatant to CML assay.

Untreated sample.

1. Centrifuge at 1000 rpm x 10 minutes.
2. Apply 50 µl of supernatant to CML assay.

APPENDIX L

CRP Procedure

CRP Procedure: (Alpco)

Reagents:

Bring reagents to room temperature and mix well.
Kit can be used up to 4 times within expiration date.
Reagents with a volume of less than 100µl should be centrifuged before use.

Wash Buffer: (Stable at 2-8 degrees C for 1 month)

- Dilute 100 ml wash buffer concentrate with 900 ml of distilled water (10x dilution).

Conjugate:

Dilute conjugate 100x in 1x wash buffer. (100 µl conjugate + 9900 µl WB). **(Diluted conjugate should not be stored.)**

Samples: (Must be diluted 1:100-1:500 before performing the assay)

- Thaw. Then immediately place on ice.
- Aliquot samples.
- Label microcentrifuge tubes.
- Add 10 mcl of sample to 990 µl sample buffer (1:100 dilution). Mix well by vortexing.

Assay:

4. Label/plan your adiponectin template.

First Wash

5. Wash with 250 µl WB per well x 5.
6. Decant the plate. Strike against towels after final wash.

First Incubation:

3. Add 100 µl of standard, sample and control in duplicate into each well.
4. Cover with a plate sealer and incubate for 1 hour at room temperature on a horizontal mixer.

Second Wash:

1. Discard contents of wells.
2. Wash with 250 µl of WB per well x 5.
3. Decant the plate. Strike against towels after final wash.

Second Incubation:

3. Add 100 µl of conjugate into each well.
4. Cover with a plate sealer and incubate for 1 hour at room temperature.

Third Wash:

4. Discard contents of wells.
5. Wash with 250 µl of WB per well x 5.
6. Decant the plate. Strike against towels after final wash.

Third Incubation

1. Add 100 µl of substrate into each well.
2. Cover with a plate sealer and incubate for 10-20 minutes at room temperature in the dark.

Stop:

3. Add 50 mcl stop reagent to each well.
4. Read at 450 nm immediately. Use a reference wavelength of 630 nm.
   If the highest standard has an OD that is overflow (S6), read the absorption at 405 nm.

APPENDIX M

Curriculum Vitae

CURRICULUM VITAE

Kathleen Davis

Education

Ph.D.	To Complete 5/2013	Nutrition, Texas Woman's University
M.S.	2001	Nutrition, Texas Woman's University
B.S.	1998	Dietetics, Texas Woman's University
B.A.	1995	French, University of North Texas

Dissertation

Dietary AGE, Diet, BMI, and Serum AGE/sRAGE: Links in a Chain?, May 2013, Victorine Imrhan

Professional Certification/Licensure:
Registered Dietitian, Licensed Dietitian, Board Certified Specialist, Pediatric Nutrition

Employment
Spring 2013, University of North Texas, Adjunct Instructor: Principles of Nutrition

Fall 2012 to Present, Quality Matters, Quality Matters Course Reviewer

August 2012 to Present, Cook Children's Medical Center, Outpatient Dietitian

Fall 2007 to Present, Owner Kathleen Davis, MS/RD/LD, Dietitian Sole Proprietorship/Nutrition Private Practice

Summer 2009-Fall 2009, Summer 2010-December 2012, Texas Woman's University, Graduate Assistant

Fall 2008-Spring 2009, Texas Woman's University, Visiting Clinical Instructor

Spring 2005, Spring 2007, Spring 2008, Texas Woman's University, Adjunct Clinical Instructor

Fall 2002, Tarrant County College, Adjunct Instructor

November 2005-full-time until October 2007; PRN until April 2011, Texas Health Presbyterian Hospital Denton, Clinical Dietitian

June 2002-November 2005, Cook Children's Medical Center, Outpatient Dietitian

April 2001-June 2002, Harris Methodist Hospital, Ft. Worth, Clinical Dietitian
January 2000-April 2001, Gainesville Memorial Hospital, Director, Dietary Services and Clinical Dietitian

TEACHING

Courses Taught at TWU
Developed and/or revised to meet Quality Matters criteria: three on-line courses:
NFS 3063 Ecology of Food and Nutrition
NFS 3173 Culture and Food
NFS 3073 Nutrition for Women in a Global Environment.

Teaching:
NFS 1302 Principles of Food Preparation
NFS 2013 Personal Nutrition
NFS 2343 Nutritional Management for the Family and Child
NFS 3033 Nutrition Throughout the Life Cycle
NFS 3043 Community Nutrition
NFS 3073 Nutrition for Women in a Global Environment
NFS 3173 Culture and Food
NFS 3713 Quantity Food Production and Service
NFS 3722 Quantity Food Production and Service Lab
NFS 5363 Human Nutrition and Disease

Other Scholarly Teaching, Mentoring and Curricular Achievements

- Certified Quality Matters Peer Reviewer for on-line courses.
- Developed guidelines for measuring goal attainment for Global Perspectives criteria for three courses in preparation for SACS accreditation. Developed measurable SLOs for undergraduate courses.

SCHOLARSHIP/CREATIVE ACHIEVEMENTS

Refereed Publications or Other Creative Achievements

Davis K, Imrhan V. Inclusion of cottonseed oil in a 5 day high fat diet regimen lowers total and LDL cholesterol in normo-cholesterolemic, healthy participants. (Published on-line: *Nutrients July 2012.*)

Davis K, Prasad C, Imrhan V. Advanced Glycation End Products, Inflammation, and Chronic Metabolic Diseases: Links in a Chain? (Accepted for publication: *Critical Reviews in Food Science and Nutrition*, October 2012).

Presentations at Professional Meetings

Poster Presentations:
Prasad C, Davis K, Imrhan V. Relationship between soluble receptor for advanced glycation endproducts (sRAGE) and adiposity: a preliminary study. International Congress on Abdominal Obesity 2012.

Davis K, Imrhan V, Prasad C. Soluble Receptor for Advanced Glycation Endproducts (sRAGE) Correlates Inversely with Body Mass Index (BMI) and Waist Circumference and Positively Correlates with High Molecular Weight Adiponectin. Experimental Biology 2012.

Davis K, Akuailou E, Prasad C, Sodhani S, Vijayagopal P, Imrhan V. Cottonseed oil rich diet reduces low density cholesterol and total cholesterol while maintaining high density cholesterol and triglycerides in healthy, adult participants. FASEB J, 2011: A38.

Davis K, Imrhan V. Cottonseed oil rich diet reduces low density cholesterol and total cholesterol while maintaining high density cholesterol and triglycerides in healthy women. Texas Dietetic Association FNCE 2011.

Davis K, Imrhan V. Effect of Participation in a University Nutrition Course with a Body Image Curriculum on Body Image, Drive for Thinness, and Success in Meeting Health Goals. Texas Dietetic Association FNCE 2002.

Invited Speaker:
Invited Speaker: "Optimizing Nutrition Education for Pediatric Weight Management" Meeting presentation at Ft. Worth Association of Nutrition and Dietetics. (November 2012)

Invited Speaker: "Effect of Cottonseed Oil (CSO) Rich Diet on Lipid Biomarkers; Cottonseed Oil: Good or Evil?" Seminar presentation at Texas Woman's University. (January 2011)

Invited Speaker: "Donor Human Milk" round table presentation at American Society of Parenteral and Enteral Nutrition Annual Conference. (February 2006, Dallas, TX)

Invited Speaker: "Nutrition in the NICU". Perinatal Nursing Symposium, Arlington, TX (Spring 2004)

Invited Speaker: "Redoing the Food Guide Pyramid". Pediatric Nursing Clinical & Prof. Update, Dallas, TX(June 2004)

Invited Speaker: "Obesity in Children" Cook Children's Pediatricians' Group, Denton, TX (February 2003)

Honors and Awards
TWU Chancellor's Research Scholar (2012)
Sarah Lucretia Cowan Endowed Scholarship (Fall 2011-Spring 2012) TWU
Phi Kappa Phi Inductee (2010)
Ardella R Helm Scholarship (2010 to present) TWU
Ima Jean Boenker cholarship (2010 to present) TWU
TWU Graduate Student Scholarship (2009 to present) TWU
Who's Who Among Students in American Colleges and Universities (2000)
Doris McCarter Brownell Scholarship (1998-1999) TWU
TWU Foundation Scholarship (1998-1999) TWU
Bobby Franklin Memorial Scholarship (1994-1996) University of North Texas
Green Jackets Scholarship (1995-1996) University of North Texas
McKenna Scholarship (1992-1993) Claremont McKenna College

Scholarly Works in Progress

Davis K, Prasad C, Imrhan V. Serum Soluble Receptor for Advanced Glycation Endproducts (sRAGE) Correlates Strongly with Measures of Adiposity in Young Adult Subjects. (In Review Acta Diabetologica February 2013).

Davis K, Prasad C, Vijayagopal P, Juma S, Warren C, Imrhan V. High Fat, High AGE Diet Promotes Rise in Serum CML. (In progress, to be submitted to journal to be identified by May 2013.)

Other Research and Creative Achievements

Quality Matters Reviewer (2012): Publisher-based course, Ashford University courses, and other reviews as needed as Subject Matter Expert and as Design Expert.

Cenage Learning Reviewer (2009): Lactation and Infant Nutrition chapters of *Nutrition Throughout the Life Cycl*e by Brown.

American Dietetic Association Reviewer (2008): Cardiovascular Disease chapter of the ADA Nutrition Care Manual

Texas Dietetic Association Reviewer (2008): Pregnancy and Lactation chapter of the TDA Nutrition Care Manual.

Nursing in Pediatrics (2004), publication of Cook Children's Medical Center.
Wrote segment on exercise for "The Three Components of Pediatric Weight Management: How to Help Children (and Their Parents) Live Healthier Lives".

Quoted in article for Dallas/Ft. Worth Child Magazine: 12/2012: "When Your Child Wants to be a Vegetarian". http://www.dfwchild.com/Dallas/showarticle.asp?artid=2007

Featured in news story on Fox 4 News (DFW) 9/17/2012: "Kids Eat Too Much Salt, as Much as Adults". http://www.myfoxdfw.com/story/19566003/cdc-us-kids-eat-too-much-salt-as-much-as-adults

Regular blog contributor to wedoitallforkids.com

PROFESSIONAL SERVICE

Service to the Profession

Graduate Club, Texas Woman's University Nutrition and Food Sciences
President
(September 2011 to August 2012)

North Texas Dietetic Association
Committee Leader: Silent Auction Baskets for TDA
(Spring 2007)

Service to the Community

Down Syndrome Camp EASE: Eating, Activity and Supported Environment
(Spring 2012)
Assisted with development of research method and IRB for proposed camp slated for kick-off in Summer 2012. Performed menu analysis for Down Syndrome camp. Worked with chef to edit kid-friendly, healthy meals. Developed curriculum to use with kids in the camp.

Lay Teaching Committee Member at First United Methodist Church
(Spring 2011)
Provided feedback and support for a theological intern in Christian Education from SMU Perkins School of Theology.

North Texas Milk Bank.
(January 2004-November 2005)
Member of the Medical Advisory Board.

Denton County Friends of the Family, Denton, TX
(January-April, 1997, 1998, 1999, 2000, 2001)
1997 – 1998 Committee Leader; 1999 – 2000 Co-Chair, Speaker, wrote article published in *Dallas Morning News*

DFW HIV/AIDS Nutrition Alliance, Dallas TX
(January 2000-June 2002)
Provided free nutrition counseling to HIV positive clients.