Vlassopoulos, Antonios (2014) *Drivers of redox status & protein glycation.* PhD thesis.

http://theses.gla.ac.uk/5607/

Glasgow Theses Service
http://theses.gla.ac.uk/
theses@gla.ac.uk

DRIVERS OF REDOX STATUS & PROTEIN GLYCATION

Antonios Vlassopoulos

BSc, MSc

A thesis submitted for the degree of Doctor of Philosophy

to

The University of Glasgow

April, 2014

From research conducted at the

School of Medicine, Human Nutrition

University of Glasgow

Glasgow, Scotland

To my aunt who showed me the way

Abstract

Background: In the past 60 years, the median age of the entire world population has increased and ageing and chronic diseases are now the main medical concerns in the developed world. The identification of early signs of disease pathogenesis is vital for prevention and targeting populations at risk in order to reduce morbidity and mortality. Glycation is well-established as an index of control, or otherwise, and a predictor of end-organ damage, for people with type 2 diabetes. At the beginning of the work for this thesis (2010), evidence was beginning to be raised to suggest that since glycation levels vary considerably, in normoglycaemic, non-diabetic individuals, glycation cannot be solely related to glucose levels and early reports allowed for speculations about a relationship between early glycation and oxidative stress. The aim of this thesis was to establish the relationship between early glycation and oxidative stress in normoglycaemia using techniques from the full breadth of Human Nutrition Research.

Methods: In study 1, existing epidemiological data were used to identify relationships between proxies for redox status and early glycation in non-diabetic individuals. One-way ANOVA, Chi-squared and multivariate linear regressions, adjusted for all known confounders were used to explore associations of HbA1c with self-reported smoking status and fruit & vegetables consumptions in the Scottish Health Surveys 2003-2010, among individuals without known diabetes and HbA1c<6.5%.

In studies 2 and 3, the associations from epidemiology were explored in mechanistic laboratory studies with high physiological relevance (using physiological concentrations and conditions) to better characterise the effect of oxidative stress and antioxidants on early glycation. In study 2, bovine serum albumin (BSA), reduced BSA (mercaptalbumin) (both 40g/L), and human plasma were incubated with glucose concentrations 0-30 mM for 4 weeks at 37°C. All were tested pre-oxidized for 8 hours prior to glycation with 10nM $H_2O_{2,}$ or continuously exposed to 10nM H_2O_2 throughout the incubation period. Fructosamine was measured (nitroblue tetrazolium method) at two and four weeks. In study 3, Bovine Serum Albumin (BSA) was pre-treated prior to in vitro glycation: either no treatment (native), pre-oxidised (incubated with 10nM $H_2O_{2,}$ for 8 hours) or incubated with a mixture of phenolic acids at physiologically relevant concentrations, for 8 hours). *In-vitro* glycation was carried out in presence of i) glucose only (0, 5 or 10mM), ii) glucose (0, 5 or 10mM) plus H_2O_2 (10nM), or iii) glucose (0, 5 or 10mM) plus phenolic acids (10-160nM). Fructosamine was again measured using the nitroblue tetrazolium method. Prior to the experimental study we carried out a

systematic literature review of dietary interventions reporting plasma concentrations polyphenol metabolites, to inform the design of a physiologically relevant *in-vitro* study.

In study 4, clinical trial data and biological samples were analysed from a randomised controlled dietary advice trial in obese pregnant women, a group at risk from higher glycation and oxidative stress. Samples and data from the UPBEAT study trial (n=117) were analysed. Plasma fructosamine, plasma sRAGE, urinary Ferric Reducing Ability of Plasma (FRAP), urinary Total Phenol (TP) and urinary Advanced Oxidised Protein Products (AOPPs) were measured at $16\text{-}18^{+6}$ and $27\text{-}28^{+6}$ weeks gestation. Dietary recalls were used to calculate fruit and vegetable and polyphenol intake at the same timepoints. Data were analysed to identify associations between dietary variables and biochemical markers, as well as their relationships with diagnosis of complications. Associations between maternal variables and neonatal anthropometry were also investigated.

Results: In study 1, HbA1c was higher in smokers by 0.25 SDs (0.08%), and 0.38 SDs higher (0.14%) in heavy smokers (>20cigarettes/day) than non-smokers ($p < 0.001$ both). Smokers were twice as likely to have HbA1c in the 'pre-diabetic' range (5.7-6.4%) ($p < 0.001$, adj.model). Pre-diabetes and low grade inflammation did not affect the associations. For every extra 80g vegetable portion consumed, HbA1c was 0.03 SDs (0.01%) lower ($p = 0.02$), but fruit consumption did not impact on HbA1c, within the low range of consumptions in this population.

In study 2, oxidized BSA (both pre-oxidised and continuously exposed to H_2O_2) was more readily glycated than native BSA at all glucose concentrations ($p = 0.03$). Moreover, only oxidized BSA was glycated at physiological glucose concentration (5mM) compared to glucose-free control (glycation increased by 35% compared to native albumin $p < 0.05$). Both 5mM and 10mM glucose led to higher glycation when mercaptalbumin was oxidised than un-oxidised ($p < 0.05$). Fructosamine concentration in human plasma was also significantly higher when oxidized and exposed to 5mM glucose, compared to non-oxidised plasma ($p = 0.03$). The interaction between glucose concentration and oxidation was found to be significant in all protein models ($p < 0.05$).

In study 3, the presence of six phenolic acids with BSA during *in-vitro* glycation did not lower fructosamine formation. However, when BSA was pre-incubated with phenolic acids, significantly lower concentration of fructosamine was detected under glycoxidative conditions (glucose 5 or 10mM plus H_2O_2 10nM) ($p < 0.001$ vs. native BSA).

In study 4, women in the lowest quartile of total polyphenol intake had 8% greater fructosamine levels compared to those in the top quartile. Total polyphenol intake was negatively correlated with sRAGE levels. Diagnosis of severe preeclampsia was associated with elevated AOPPs. Maternal

polyphenol intake was positively correlated with birth weight, while maternal glycoxidation showed the opposite relationship.

Conclusions:

Study 1 added evidence to relate smoking (an oxidative stress proxy) to protein glycation in normoglycaemic subjects. This association has implications for individuals exposed to ROS and for epidemiological interpretation of HbA1c and its clinical usefulness.

Study 2 offered a mechanistic background to the previously shown epidemiological association. This study demonstrated for the first time albumin glycation *in-vitro*, using physiological concentrations of albumin, glucose and hydrogen peroxide. These results identified low-grade oxidative stress as a key element early in the glycation process, especially in glucose concentrations relative to normoglycaemia. Furthering those findings, study 3 showed that protein-phenolic acid interactions are important regulators of protein glycation. Together those studies highlighted that protein pre-treatment, either with oxidants or phenolic acids, is an important regulator of subsequent glycation in a physiologically relevant system. An important outcome of those studies is that high quality *in-vitro* studies under conditions closer to physiology are feasible and should be employed more frequently.

Finally, study 4 demonstrated an association between polyphenol intake and glycation during pregnancy, with an impact on neonatal outcome measures. Maternal glycoxidation is a promising marker of pre-eclampsia and neonatal anthropometry and could be modulated by maternal lifestyle and dietary habits.

Overall, the results of this thesis implicate that drivers of redox status have the capacity to modulate protein glycation in normoglycaemia. These results challenge the assumption that glycation levels are solely dependent on circulating glucose levels and suggest a useful application of glycation outside the field of diabetes.

Acknowledgements

I want to thank my supervisors Dr Emilie Combet and Prof Mike Lean for all the help and outstanding support all along the way. They believed in me from the beginning and pushed me to become better. It is their efforts that helped become who I am today and that I will remember and value throughout my life. It was my pleasure to work and learn from you and I hope that this is not the end for our collaboration. I like to think that along the way we've built a relationship that goes beyond the strictly professional limits and that I would be very sorry to lose.

I would like to thank the Yorkhill Children Charity, for providing the funding for this PhD, and also Prof Scott Nelson and Prof Lucilla Poston for kindly offering us access to the UPBEAT samples and data.

A special thank you goes to all the laboratory and administrative staff in the Department of Human Nutrition, for dealing with all my bizarre and last minute requests. Also to Mr Philip Mcloone for his guidance in analysis data and dealing with large datasets.

I should also thank the academic staff of the Department for they entrusted me with teaching lab practicals, journal clubs and all sorts of things to our MSc students from year to year. I hope I did a good job and you are not regretting your choice.

The help of our interns and students involved in any part of my projects is highly appreciated and I should thank you for that.

A big thank you goes to all the people that were next to me day after day. They were there when I laughed, complained and felt like giving up. Jeremy Jones, Areej Alkhaldy, Martin Bertz and Friederike Vollmer-Bertz thank you for everything.

My very close friend Bahareh Mansoorian, who is submitting today with me, we've gone a long way with ups and downs but we made it at the end.

I've left for the end those that worked like my safety net/my guardians, my family, my aunts and Ilektra. You were always my last resort, the place I knew I would find safety and peace, there not words big enough to thank you.

Author's Declaration

I declare that the work contained in this thesis is original, and is the work of the author Antonios Vlassopoulos. I have been solely responsible for the organisation and day to day running of this study as well as the laboratory analysis and data processing, unless otherwise referenced.

Antonios Vlassopoulos

Supervisors' declaration

I certify that the work reported in this thesis has been performed by Antonios Vlassopoulos and that during the period of study he has fulfilled the conditions of the ordinances and regulations governing the Degree of Doctor of Philosophy, University of Glasgow

Dr. Emilie Combet Aspray   Prof. Michael EJ Lean

Table of Contents

List of Tables

List of Figures

Publications achieved during the present thesis

Full papers

Vlassopoulos A, Lean MEJ, Combet E. (2014) Inhibition of protein glycation by phenolic acids: physiological relevance and implication of protein-phenolic interactions. *Food & Function* (accepted)

Vlassopoulos A, Lean MEJ, Combet E. (2014) Oxidative stress, protein glycation and nutrition - interactions relevant to health and disease throughout the lifecycle. *Proceedings of the Nutrition Society* (accepted)

Vlassopoulos A, Lean MEJ, Combet E. (2013) Role of oxidative stress in physiological albumin glycation: A neglected interaction. *Free Radical Biology and Medicine* **60**, 318-324.

Vlassopoulos A, Combet E, Lean MEJ. (2013) Changing distributions of body size and adiposity with age. *International Journal of Obesity*

Vlassopoulos A, Lean MEJ, Combet E. (2013) Influence of smoking and diet on glycated haemoglobin and'pre-diabetes' categorisation: a cross-sectional analysis. *BMC Public Health* **13**(1), 1013

Abstracts

Vlassopoulos A, Combet E, Lean MEJ. (2013) Evidence for continuing body-fat accumulation into old age. *Obesity Facts* **6**(suppl 1), 223

Vlassopoulos A, Lean MEJ, Combet E. (2013) Role of oxidative stress in physiological albumin glycation: a neglected link. *Diabetic Medicine* **30**, E2-E2

Combet E, Vlassopoulos A, Lean MEJ. (2013) Influence of smoking and diet on glycated hemoglobin and 'pre-diabetes' categorization. *Diabetic Medicine* **30**, E6-E7

Vlassopoulos A, Lean MEJ, Combet E. (2012) The role of oxidative stress on protein glycation. *Proceedings of the Nutrition Society* **71**(OCE2), E57

List of Abbreviations

ADA	American Diabetes Association
AGE-R1	AGE-Receptor complex 1
AGE-R2	AGE-Receptor complex2
AGE-R3	AGE-Receptor complex 3
AGEs	Advanced Glycation Endproducts
ANOVA	Analysis of Variance
AOPPs	Advanced Oxidation Protein Products
AOS	Action on Smoking
ARIC	Atherosclerosis Risk in Communities study
AusDiab	Australian Diabetes, Obesity and Lifestyle Study
BMI	Body Mass Index
BSA	Bovine Serum Albumin
CD36	Cluster of Differentiation 36
CHD	Coronary Heart Disease
CML	N-Carboxymethyllysine
CRP	C-Reactive Protein
CV	Coefficient of Variation
CVD	Cardiovascular Disease
DCCT	Diabetes Control and Complications Trial
DMF	Deoxy-1-Morpholinofructose
DNA	Deoxyribonucleic acid
DTT	Dithithreitol
ELISA	Enzyme Linked Immunosorbent Assay
EPIC	European Investigation in Cancer and Nutrition
ESDS	Economic and Social Data Service
F&V	Fruit & Vegetable
FAO	Food and Agriculture Organisation
FFQ	Food Frequency Questionnaire
FRAP	Ferric Reducing Ability of Plasma
GDM	Gestational Diabetes Mellitus
GI	Glycaemic index
GSH	Glutathione
HbA1c	Glycated Haemoglobin
HPLC	High Pressure Liquid Chromatography
HSCIC	Health and Social Care Information Centre
ICAM-1	Intercellular Adhesion Molecule 1
IgG	Immunoglobulin G
IL-1β	Interleukin-1-beta
IL-6	Interleukin-6
JIA	Juvenile Idiopathic Arthritis
LDL	Low-Density Lipoprotein
LGA	Large for Gestational Age
LOX-1	Lectin-like oxidised low-density lipoprotein (LDL) receptor-1

MGO	Methylglyoxal
MONICA	Monitoring of Trends and Determinants in Cardiovascular Disease
MS	Mass Spectrometry
MSRII	Macrophage Scavenger Receptor II
MUFA	Monounsaturated Fatty Acids
MWCO	Molecular Weight Cut-off
NAD(P)H	Nicotinamide Adenine Dinucleotide Phosphate
NBT	Nitroblue Tetrazolium
NCD	Non-Communicable Diseases
NFκB	Nuclear Factor Kappa-light-chain-enhancer of activated B cells
OGTT	Oral Glucose Tolerance Test
OR	Odds Ratio
ox-BSA	Pre-oxidised BSA
PBS	Phosphate Buffer Saline
PP-BSA	Phenolic acid-preincubated BSA
PRISMA	Preferred Reporting Items for Systematic Review and Meta-Analysis
PUFA	Polyunsaturated Fatty Acids
Q1	Quartile 1
Q4	Quartile 4
RAGE	Receptor for AGEs
RCT	Randomised Controlled Trial
ROS	Reactive Oxygen Species
SD	Standard Deviation
SGA	Small for Gestational Age
SHS	Scottish Health Survey
sRAGE	Soluble Receptor for AGEs
TBARS	Thiobarbituric Acid Reactive Substances
TBH	t-Butyl Hydroperoxide
TNF-α	Tumour Necrosis Factor- alpha
TP	Total Phenol
UK	United Kingdom
UPBEAT	UK Pregnancies Better Eating and Activity Trial
US	United States
VCAM-1	Vascular Cell Adhesion Molecule 1
WC	Waist Circumference
WHO	World Health Organisation
WHS	Women's Health Survey
8-OH-dG	8-hydroxydeoxyguanosine

Chapter 1: Introduction

This chapter is presented as published in the Proceedings of the Nutrition Society

Winner of Postgraduate Competition,

Nutrition Society Summer Meeting 2013, University of Newcastle

1.1 General Introduction

In the modern world non-communicable diseases (NCD) are the greatest public health concern. In 2008, 63% of global deaths, 36 million deaths in numbers, were caused by one of the four major NCDs; diabetes, cardiovascular disease, cancer and chronic respiratory diseases (WHO, 2011). Even though at the moment in most places in the world, NCD mortality is higher than that of maternal, neonatal, perinatal, communicable and nutritional condition mortality combined, it is far from reaching a plateau. It is estimated that by 2020, NCD deaths will have increased globally by 15%, with low-to-middle income countries seeing the greatest increase (over 20%) and the European region remaining generally unaffected (WHO, 2010).

The burden of NCD deaths is disproportionately affecting different parts of the world. The majority (80%) of NCD deaths caused by diabetes or cardiovascular disease occur in low-to-middle income countries, while at the same time 29% of total NCD deaths in those countries are likely to happen before the age of 60 (WHO, 2011). In developed countries, on the other hand, more and more people are living daily with NCD risk factors, as the population group of over 60 is the fastest growing population group in the developed world and early NCD deaths are only 13% of the total (WHO, 2014).

NCDs have been causally linked to four major risk factors: alcohol overuse, tobacco smoking, unhealthy diet and inactivity. These behaviours lead to four common (metabolic) conditions: high blood pressure, overweight/obesity, hyperglycaemia and raised cholesterol, all identified as common underlying causes of NCDs. Raised blood pressure has been attributed 13% of global deaths, while tobacco smoke and overweight/obesity are responsible for 9% and 5% of global deaths, respectively. As far as unhealthy diet is concerned 1.7 million deaths worldwide (~3%) are attributable to low fruit and vegetable consumption (WHO, 2011). Data from the European Investigation in Cancer and Nutrition (EPIC) showed that consuming ≥5 portions of fruit and vegetable per day was associated with a nearly 10% lower risk of diabetes (Cooper et al., 2012). Similarly an investigation into three of the largest US prospective studies the Nurse's Health Study, Nurse's Health Study II and the Health Professional's Follow-up study showed that every 3-portion increment in the consumption of blueberries, grapes and apples was associated with 26%, 12% and 7% lower diabetes hazard ratio respectively (Muraki et al., 2013). Other prospective studies have also shown apples to be associated with a 28% reduction in the risk of diabetes from consumptions as little as 1-apple a day (Song et al., 2005). More interestingly a recent report calculated, using existing data, that consuming one apple daily is associated with lower vascular mortality of those aged over 50 years, with a net gain of 8500 lives annually (Briggs et al., 2013).

Interventions aiming to reduce the prevalence of NCD risk factors and the development of screening to identify people at risk of NCDs early in the lifecourse have been proposed by the WHO as cost-effective solutions to a growing public concern (WHO, 2011).

Inflammation is a common trait for all the NCD risk factors. Large scale cross-sectional investigations, like the MONICA (Multinational **MONI**toring of trends and determinants in **CA**rdiovascular disease) Survey have showed a significant positive correlation between smoking and inflammation markers like C-Reactive Protein (CRP), white blood cell count and fibrinogen in men (Fröhlich et al., 2003). Increased levels of CRP, smoking and features of metabolic syndrome have all been independently associated with increased risk of hypertension (Niskanen et al., 2004) and having IL-6 in the highest tertile was associated with a 60% increase in the risk of developing diabetes as a non-smoker compared to those in the lowest tertile (Duncan et al., 2003). Similarly obesity and especially central adiposity, both NCDs risk factors, have been strongly associated with CRP levels (Festa et al., 2001, Forouhi et al., 2001, Yudkin et al., 1999). Visceral and subcutaneous adipose tissue as measured with tomography were also significantly associated with CRP and IL-6 levels in the Framingham study, which indicated a positive correlation with oxidative stress as well (Pou et al., 2007). Other groups have also found a positive correlation between oxidative stress levels and NCDs risk factors like smoking, obesity and low fruit and vegetable intake, showcasing its importance as another potential common trait in NCDs pathogenesis (Isik et al., 2007, Marangon et al., 1998, Keaney et al., 2003, Holt et al., 2009, Block et al., 2002, Mayne, 2003, Sies et al., 2005).

Oxidative stress has been identified as a unifying mechanism leading to disease pathogenesis. In the healthy state, Reactive Oxygen Species (ROS) are formed constantly through cell respiration, immune mechanisms and metabolic pathways, but their levels are tightly control by endogenous antioxidant systems, like glutathione peroxidase (Rizzo et al., 2010). In the process, though, of disease pathogenesis there is increased production of ROS which overwhelms the endogenous antioxidant systems (Juranek and Bezek, 2005). The imbalance between antioxidant defences and ROS production is called oxidative stress. Inflammation, obesity, tissue hypoxia and lifestyle parameters like smoking and low physical activity have all been associated with higher oxidative stress (Hulsmans and Holvoet, 2010, Bondia-Pons et al., 2012, Peng et al., 2003, Lavie, 2009, Wood et al., 2009). At the stage where endogenous antioxidant systems are overwhelmed dietary intake of antioxidant compounds, like vitamins, minerals and (poly)phenols may be of importance in maintaining a better regulated redox status (Pham-Huy et al., 2008, Rodrigo et al., 2011, Serafini and Del Rio, 2004, Sies et al., 2005).

As most NCDs are diseases related with the ageing process, glycation and the Maillard reactions have attracted a fair amount of attention as a pathogenic mechanism of NCDs (Lyons, 1993,

Geroldi et al., 2005, Zieman et al., 2007). Glycation is a process central to ageing and strongly related with oxidative stress and inflammation (Wolff et al., 1991, Ramasamy et al., 2005). Biopsies from liver, fat and muscle tissues have shown accumulation of glycation products in obesity and during weight gain (Gaens et al., 2012, de la Maza et al., 2008). Also experimental data on adipocytes, suggest that glycation products are deposed intra-cellularly as part of the adipogenesis process and this deposition is associated with increased oxidative stress and inflammation (Gaens et al., 2013, Chen et al., 2012). Early reports also suggest that smoking is associated with higher levels of glycation and that diet may also be playing a role in the levels of circulating glycation products (Nicholl and Bucala, 1998, Harding et al., 2004, Sebekova et al., 2001). Western type diets, high in fried and processed foods, are a good source of Advanced Glycation Endproducts (AGEs) produced during heat-treatment of food items (Uribarri et al., 2010, Goldberg et al., 2004). These dietary/exogenous AGEs contribute towards the circulating AGEs pool and have been shown to increase systemic inflammation and oxidative stress (Uribarri et al., 2005, Šebeková and Somoza, 2007), but there insufficient evidence so far to support any health benefit following a low-AGE diet (Kellow and Savige, 2013).

1.2 The glycation reaction - historical background

Glycation, also called non-enzymatic browning or the Maillard reactions, has attracted scientific interest for nearly a century. Initiated by the non-enzymatic condensation of a reducing sugar (like glucose) with a protein, glycation is one of the most important forms of protein damage, relevant to both medicine and food science. Not be confused with enzymatic glycosylation, a tightly regulated process that attaches carbohydrates to specific sites on the protein molecule (Darnell et al., 2000, Moharir et al., 2013, Spiro, 2002), glycation is a form of protein damage reducing functionality (Hunter et al., 2003) while glycosylation is an essential post-translational modification required for the molecule to function (Darnell et al., 2000, Moharir et al., 2013, Spiro, 2002).

Named after the pioneer in the field, the Maillard reactions were described in 1912 (Maillard, 1912). The exact chemical transformations taking place as part of the Maillard reactions are complex and, despite years of research, still poorly understood. The early work of Louis Camille Maillard described the theoretical background for the reactions, which attracted interest from food scientists, as relevant to aroma and flavour development during cooking, as well as a cause of protein loss during storage and cooking. The reactions were not properly described until John E. Hodge proposed a mechanism of action in three stages: 1) the carbonyl group of a sugar reacts with the amino group of a protein/amino acid to produce an unstable Schiff base, 2) the Schiff

base undergoes Amadori rearrangements to produce the first stable product, a ketoamine (Amadori, 1931, Hodge, 1955) and 3) the ketoamine, undergoes further rearrangements, polymerization and condensations leading to the production of a broad range of compounds , collectively termed AGEs (Hodge, 1953). In the same step, AGEs undergo further degradation, polymerisation and oxidation to produce high molecular weight polymers called melanoidins, which are still poorly characterised and understood (Ledl and Schleicher, 1990, Wang et al., 2011) (Figure 1.1).

The concept of *in-vivo* protein glycation was later discovered in 1977, when HbA1c, a fraction of haemoglobin shown to migrate slower during electrophoresis, was identified as a ketoamine, specifically a fructosamine (Koenig et al., 1977). Upon its discovery, HbA1c was proposed as a useful biomarker for diabetes monitoring (Koenig et al., 1976a, Koenig et al., 1976b), and endogenously produced AGEs attracted further scientific attention, beyond food chemistry, from fields including medical biochemistry and pathology. At this point it is important to highlight that there are two sources of AGEs: the endogenously produced AGEs, a product of the reaction between proteins and sugars in the circulation, and exogenous AGEs, produced during cooking and absorbed in the small intestine (~10% bioavailability).

Figure 1.1. Brief overview of the Maillard reactions
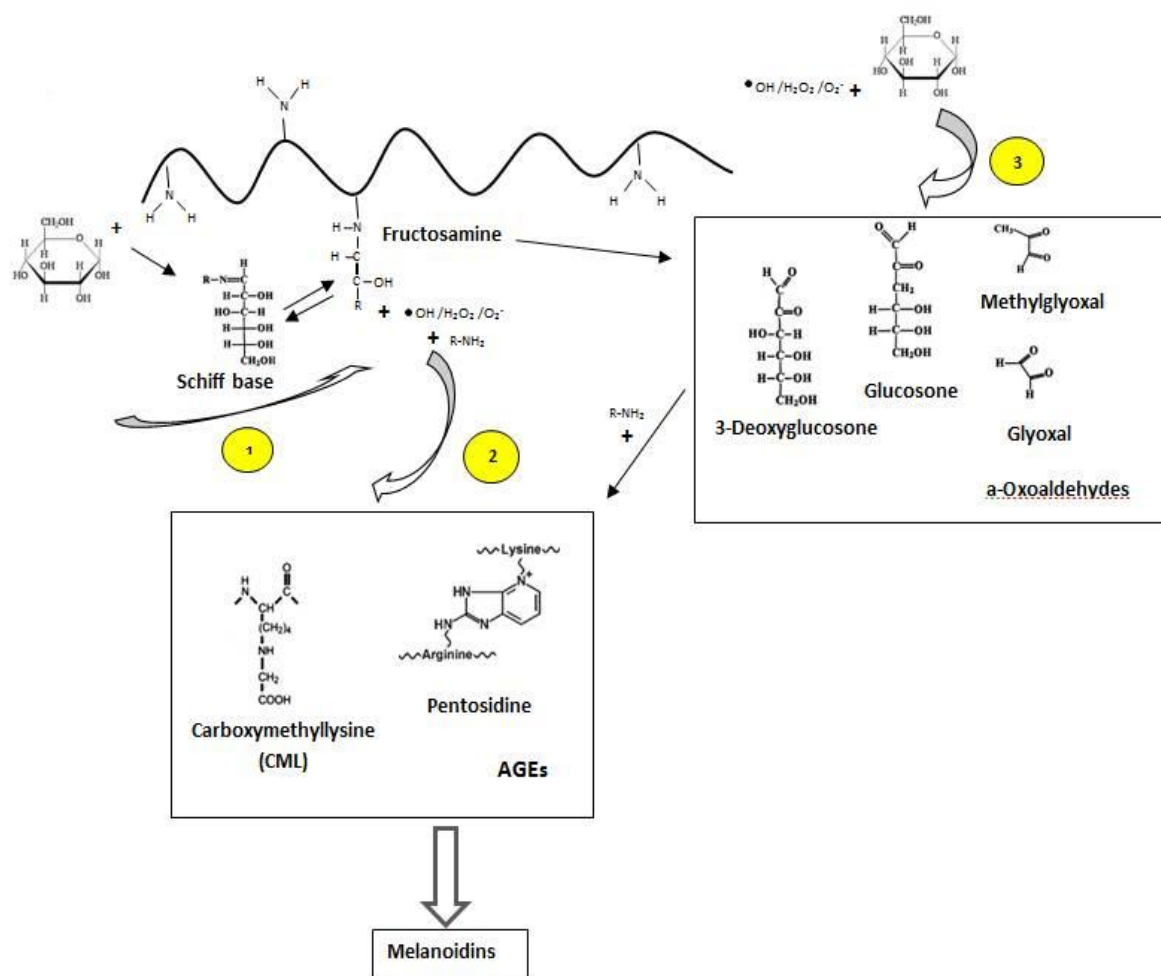
The figure describes the main pathways of glycoxidative AGEs production. In pathway 1, a sugar reacts with the amino group of a protein to form a Schiff base, which will then undergo Amadori rearrangement to produce the first stable product, a ketoamine (in the case of glucose being the reducing, a fructoseamine). This represents stages 1 and 2 in the model proposed by Hodge (1953). In pathway 2, the ketoamine undergoes oxidative fragmentation and forms the two main AGEs, carboxymethyllysine and Pentosidine. Then the AGEs enter another final step of reactions to produce a class of poorly understood compounds, melanoidins. Pathway 2 and the melanoidin production step represent the third stage of the Maillard reactions according to Hodge. Pathway 3, depicts the glucose autoxidation or Wolff pathway(Wolff and Dean, 1987). In that, glucose is autoxidised in the presence of free radical to produce a-oxoaldehydes. These a-oxoaldehydes can then react further with proteins to produce AGEs. a-oxoaldehydes can also be produced from ketoamine degradation and lipid peroxidation (not shown in the figure).

1.3 The importance of glycation for health

1.3.1 Glycation and the AGE-RAGE axis

The study of the role played by glycation in disease pathogenesis originally relied on measuring fructosamine levels in biological fluids combined with the characterisation of endogenous AGEs in the circulation and tissues (Schmidt et al., 1994, Sell and Monnier, 1989a). The discovery that AGEs can bind on cellular receptors and alter intracellular events was a breakthrough (Schmidt et al., 1992), linking glycation to signalling. These receptors include AGE-R1, AGE-R2, AGE-R3, MSRII, CD36, LOX-1 and the Receptor for AGEs (RAGE), the most characterised receptor (Vlassara et al., 1995, Maillard-Lefebvre et al., 2009). RAGE is a multi-ligand cell-surface immunoglobulin, with the ability to initiate injury-like intracellular events, mainly expression of genes related with inflammation and oxidative stress (Schmidt et al., 1995, Hofmann et al., 1999, Kislinger et al., 1999). Together, accumulation of AGEs in tissues and their interaction with cell surface receptors are the main mechanisms that explain glycation-related pathophysiology to date.

These discoveries were accompanied by the study of systems in place to reduce glycation-related damage, further contributing to the understanding of pathophysiological mechanisms. One such system is the glyoxalase system that neutralises α-oxoaldehydes, oxidatively produced AGEs precursors, and inhibits oxidative stress related glycation (Thornalley, 2003b). Similarly, fructosamine-3-kinase, which removes ketoamine moieties from proteins, is able to reverse the second stage of the Maillard reactions (Delpierre et al., 2000). Finally, sRAGE, the soluble form of RAGE, can bind on AGEs and may act as a decoy, preventing AGEs from interacting with RAGE (Raucci et al., 2008, Lindsey et al., 2009, Koyama et al., 2007, Maillard-Lefebvre et al., 2009, Selvin et al., 2013).

Accumulation of AGEs in tissues and AGE-RAGE interactions are the two main pathways of glycation involvement in disease pathogenesis. These two pathways are often acting simultaneously and their individual effects are hard to distinguish; hence they are commonly presented in the same context when discussing glycation related pathophysiology (Takeuchi and Yamagishi, 2008, Munch et al., 2012, Brownlee et al., 1988, Zhang et al., 2009, Stitt, 2001, Creager et al., 2003).

In hyperglycaemia (post-prandially or in non-controlled diabetes) and to a lesser extent in normoglycaemia, both circulatory proteins and proteins of the endothelium are exposed to (excess) glucose, leading to the slow formation of AGEs (Monami et al., 2006, Landgraf, 2004, Ahmed et al., 2005, Beisswenger et al., 2001). Long-lived proteins, such as collagen and elastin,

are the molecules most affected by glycation. In the presence of glucose, glycation adducts are created on the collagen molecule, as a function of glucose levels. Those adducts promote excessive cross-linking among collagen molecules, inhibiting the formation of an ordered and functional polymeric complex. These changes lead to the formation of a thick vascular wall with i) reduced elasticity (or increased intermolecular pore size of the basement membrane in kidneys) and ii) a high affinity of collagen for other circulating proteins like IgG, albumin and lipoproteins like Low Density Lipoprotein (LDL) (Brownlee et al., 1988, Lepape et al., 1981, Sensi et al., 1986, Vlassara, 1996, Sell and Monnier, 1989b, Monnier, 1989, Bobbink et al., 1997, Kent et al., 1985, Tanaka et al., 1988, Sensi et al., 1989). The immobilisation of proteins onto the vascular wall creates a vicious cycle where these proteins are becoming more susceptible to glycation (reduced removal by enzymes-increased half-life) and to further crosslinking. Once glycated, these proteins act as chemo-attractors for macrophages and monocytes, promoting inflammation and 'foam' cell formation in the endothelium (Klein et al., 1995, Iwashima et al., 2000, Basta et al., 2004). Glycated molecules like LDL and insulin also display lower affinity to their receptor, with a reduced ability to initiate receptor mediated events, like glucose uptake (Abdel-Wahab et al., 1997, Monnier, 1989, Berrou et al., 2009, Duran-Jimenez et al., 2009).

RAGE mediated events are also central to the role of glycation in pathophysiology. Upon activation of RAGE, intracellular ROS levels are increased through the NAD(P)H oxidase. This in turns leads to the activation of the Ras-MAP kinase pathway, ultimately upregulating NFκB and the production of inflammatory molecules (including TNF-α, VCAM-1, I-CAM1 and IL-1β). The upregulation of NFκB also initiates a positive feedback loop that sensitises the cell (and hence the tissue) to AGEs by promoting RAGE production (Basta et al., 2004).

1.3.2 Glycation and health throughout the lifecycle

Glycation is relevant to all stages in the lifecycle, including conception and early gestation. The reproductive tract is a known target of AGEs accumulation both in men (Mallidis et al., 2009) and women (Diamanti-Kandarakis et al., 2007). AGEs accumulation is followed by changes in the distribution of RAGE in reproductive tissues (Mallidis et al., 2007), and sRAGE in seminal/follicular fluid (Karimi et al., 2012, Bonetti et al., 2013), which may lead to lower sperm quality (Karimi et al., 2012), lower likelihood of success following assisted reproduction (Malickova et al., 2010, Jinno et al., 2011) and reduced embryonal quality and development (Jinno et al., 2011, Bonetti et al., 2013, Hao et al., 2008). During the course of pregnancy, activation of the AGE-RAGE axis may be involved in the pathogenesis of preeclampsia (Oliver et al., 2011, Naruse et al., 2012, Cooke et al., 2003).

So far evidence on the involvement of AGEs and/or RAGE in fetal development are limited and based on animal studies. For example a study on transgenic mice showed that overexpression of RAGE was associated with impairements of alveolar morphogenesis. The degree of RAGE overexpression was related to the magnitude of the abnormality with homozygous mice having histological changes similar to human bronchopulmonary dysplasia. The study also found that this early life changes could lead to increased risk of 'destructive' emphysema (Fineschi et al., 2013). In humans the leading hypothesis is linking glucose/fructose related damage during diabetic pregnancies with diabetic embryopathy. In this hypothesis, glycation of proteins, lipids and DNA of the developing offspring could be one potential mechanism linking the high prevalence of malformations in babies of diabetic mothers to the high maternal blood glucose levels (Chugh et al., 2003, Vlassara and Palace, 2002).

While the role of glycation in human fetal development still needs to be documented, the child is exposed to external sources of AGEs early in life. Dietary AGEs have the potential to act as allergens or increase the immune response to known allergens. In particular, the glycated form of casein, peanut proteins and ovalbumin have been shown to increase the immunological and inflammatory responses involved in allergies (Ilchmann et al., 2010, Deo et al., 2009, Mueller et al., 2013). Glycation is a feature of juvenile idiopathic arthritis (JIA), a disease occurring in adolescence. Increased glycation, AGE accumulation through the lifecycle, and its relationship with arthritis and the disease activity, may be potentiated by early activation of RAGE in younger patients suffering from JIA (Myles et al., 2011).

Glycation has been proposed as a mechanism of ageing (Gul et al., 2009, Gkogkolou and Bohm, 2012). Evidence from animal models suggest that a diet low in AGEs (50% reduction in AGEs intake) was associated with amelioration of insulin resistance, lower AGEs accumulation (both indications of the ageing process) and ultimately increased lifespan compared to the controls (Cai et al., 2007). Similarly, mice on caloric restriction, a popular model of lifespan expansion in animal models, have lower levels of collagen cross-linking and lower levels of lens cataract, suggesting lower AGEs accumulation in the vitreous and the extracellular matrix (Taylor et al., 1995, Reiser, 1994) as well as in the brain (Mouton et al., 2009). In fact, mice fed high AGEs diets while on caloric restriction did not show any increase in their lifespan and the authors of the report suggested that lower AGEs intake may be one of the mechanisms behind the caloric restriction model (Cai et al., 2008, Cai et al., 2007). These findings seem to be universal among species and have been replicate in non-human primates, highlighting the central role of glycation in the ageing process at least in mammals (Sell et al., 2003).

Within the concept that ageing and apoptosis are timed by 'a master biological clock', AGEs accumulation has been proposed as the mechanism that sensitises this 'clock' and programmes

ageing and cellular death (Severin et al., 2013). On a cellular level, longevity is associated with decreased oxidative stress, more effective signal transaction, improved insulin sensitisation and decreased level of mutations (Sinclair, 2005). AGEs through their receptor RAGE, have the capacity to regulate NFκB activation (Nedic et al., 2013). Upregulation of NFκB has a pro-oxidant effect as it increases ROS production. It also increases the production of TNF-α, IL-6, IL-1β and there is evidence to suggest that this pathway is responsible for vascular ageing (Wautier and Schmidt, 2004, Sousa et al., 2000, Schmidt et al., 2001). DNA glycation has been shown to take place intracellularly and leads to increased number of mutations (Baynes, 2002). Finally, the glycation of intracellular proteins, like insulin, has the capacity to substantially reduce signal transaction and in this case also reduce insulin sensitivity (Abdel-Wahab et al., 1997, O'Harte et al., 1996, Oliveira et al., 2011, Hunter et al., 2003). An interesting observation linking the effect of AGEs in ageing and as early in life as in conception comes from a study showing the active involvement of AGEs accumulation in ovarian ageing and ovarian function in human subjects (Stensen et al., 2014).

1.3.3 HbA1c and risk of chronic diseases

Even though the exact mechanisms of disease pathogenesis remain elusive, extensive evidence is available to associate glycation with disease risk. Glycation has a particular relevance for age-related diseases, including Alzheimer's disease (Smith et al., 1995, Srikanth et al., 2011), skin ageing (Gkogkolou and Bohm, 2012) and cataract (Gul et al., 2009). These conditions are characterised by increased, possibly lifelong, deposition of AGEs in the affected tissue (Vitek et al., 1994, Bailey et al., 1993, Nicholl et al., 1998). The AGEs are mainly produced locally and often increased concentrations in tissue are not reflected in increased plasma levels, like in the case of Alzheimer's disease (Thome et al., 1996). Similar deposition of AGEs in the cartilage and/or increased circulating levels of AGEs is also found in rheumatoid arthritis and osteoarthritis (DeGroot, 2004, Matsumoto et al., 2007, Vos et al., 2010); collagen glycation has been proposed to be a contributor to stiffening of the micro and macro-vasculature, with implications for chronic kidney disease (Nakamura et al., 2009) and loss of kidney function in both diabetic and non-diabetic nephropathy (Tanji et al., 2000).

As *in-vivo* glycation is believed to be mainly driven by plasma glucose concentrations, the most established relationship is between glycation and diabetes. HbA1c is the gold standard method for diabetes diagnosis and monitoring (American Diabetes et al., 2007). According to the American Diabetes Association, individuals with HbA1c levels between 5.7-6.5 % are considered at high risk of developing diabetes. Those with HbA1c>6.5% are classified as having diabetes (American Diabetes, 2011). Among patients with diabetes, higher HbA1c levels are associated

with increased risk of retinopathy (Porta et al., 2001, Klein et al., 1994, Klein et al., 1998, 1995, McCarter et al., 2004), neuropathy (El-Salem et al., 2007) and nephropathy (McCarter et al., 2004). These associations led to an expert position statement describing the benefits of glycaemic control, measured as HbA1c levels, in reducing the risk for micro and macrovascular diabetic complications (Skyler et al., 2009). Similar results were reported by the UKPDS documented a 21% reduction in the risk of any diabetes related endpoint for every 1% reduction in mean HbA1c (Stratton et al., 2000). Even though HbA1c levels are traditionally associated with disease pathogenesis in diabetes, glycation has recently attracted attention as a risk factor for normoglycaemic individuals.

For the purpose of this thesis, we conducted a systematic literature search to identify studies documenting the association between glycation and the risk of non-communicable chronic diseases in normoglycaemic subjects. We identified 15 reports from 8 studies (European Prospective Investigation into Cancer and Nutrition-EPIC (Khaw et al., 2004a, Khaw et al., 2004b), Atherosclerosis Risk in Communities study-ARIC (Selvin et al., 2010, Selvin et al., 2005, Matsushita et al., 2010, Selvin et al., 2014), Australian Diabetes, Obesity and Lifestlyle study-AusDiab (Barr et al., 2009), the Hoorn Study (de Vegt et al., 1999, van't Riet et al., 2012), Framingham Offspring (Meigs et al., 2002), Rancho Bernardo (Park et al., 1996), Women's Health Study-WHS (Pradhan et al., 2007, Lin et al., 2006, Blake et al., 2004) and National Survey of Cardiovascular Disorders 1990-NIPPON DATA90 (Sakurai et al., 2013)) analysing data from a total of over 63,000 participants, followed-up for 4-15 years. The outcomes of interest were diabetes risk, cardiovascular disease (CVD), ischemic heart disease, stroke, coronary heart disease (CHD) and all-cause and CVD mortality. Two reports focused on the association between glycation and cancer risk, especially colorectal (Khaw et al., 2004b) and breast cancer (Lin et al., 2006).

Data from the ARIC and EPIC studies suggested a positive association between HbA1c levels and CVD. Higher HbA1c levels (by 1%) were associated with a nearly 40% higher risk of heart failure (Matsushita et al., 2010). Similarly there was almost a 23% higher risk for stroke between individuals in the highest HbA1c tertile compared to those in the lowest tertile in non-diabetic subjects (Selvin et al., 2005, Selvin et al., 2010). In the total population of the ARIC study, each 1% increase in HbA1c was associated with a 55% higher risk of stroke (Selvin et al., 2010). Although increased HbA1c is quite consistently shown to be related with higher risk for all-cause mortality the relationship is not necessarily linear as proposed by the analysis of the EPIC-Norfolk data (Khaw et al., 2004a). An analysis of the ARIC study data showed that having HbA1c levels lower than 5% may also be related to increased all-cause mortality suggesting a J-shaped relationship (Selvin et al., 2010). Overall the EPIC, ARIC and AusDiab studies suggested a 18-26% higher all-cause mortality among for every 1% higher HbA1c levels (Barr et al., 2009, Khaw et al., 2004a,

Selvin et al., 2010) while HbA1c levels higher by 1% were associated with 32-50% higher risk CVD death in the AusDiab and NIPPON90 investigations (Barr et al., 2009, Sakurai et al., 2013). On the contrary, the Hoorn study in elderly individuals did not show any association between HbA1c and all-cause mortality or CVD mortality, but reported a positive association between HbA1c and non-fatal CVD risk (de Vegt et al., 1999, van't Riet et al., 2012). Both reports and the results from WHS suggested that HbA1c is associated with increased risk of CVD/all-cause mortality in non-diabetic women and older participants, but this association is largely explained by the correlation between HbA1c and traditional CVD risk factors (Blake et al., 2004, de Vegt et al., 1999, Pradhan et al., 2007, van't Riet et al., 2012). Selvin et al (Selvin et al., 2010) found that the increase in CHD and all-cause mortality associated with higher HbA1c levels was not related to fasting glucose levels (Selvin et al., 2010).

As far as cancer incidence is concerned the results are still inconclusive. Data from the EPIC cohort suggest a 33% higher incidence of colorectal cancer per every 1% higher HbA1c (Khaw et al., 2004b), but an analysis of the WHS data did not find any association between HbA1c and breast cancer risk (Lin et al., 2006). As the two cancer types differ significantly in aetiology, colorectal cancer has a strong dietary link (Edwards et al., 2010) while breast cancer is mainly of genetic aetiology (McPherson et al., 2000); more research is needed before any conclusion is reached. It is important at this stage to highlight that the epidemiological associations between HbA1c levels and morbidity/mortality in both diabetics and non-diabetics may be pointing towards a direct mechanism of action, but it could also be the case that HbA1c serves a proxy marker for poorer general health.

1.4 Oxidative stress and glycation

As observed by Selvin et al (2010), fasting glucose may fail to explain the positive relationship between HbA1c and CVD and/or mortality. Correction for classical risk factors (including smoking, dyslipidaemia, inflammation) explain the relationship better (van't Riet et al., 2012, de Vegt et al., 1999, Pradhan et al., 2007, Blake et al., 2004), suggesting that a shared mechanism may drive the increase in HbA1c levels.

In the glycation pathway, oxidative stress can increase glycation in two ways, by promoting glucose autoxidation or by fructosamine oxidation leading to increased AGEs production (mainly CML). The first mechanism, the Wolff pathway, suggests that in the presence of oxidative stress, especially transition metal ions, glucose can get 'autoxidised' and produce a-oxoaldehydes (like methylglyoxal). The oxoaldehyde can react with the protein and contribute to AGEs formation;

the hydroxyl radicals can induce further oxidative damage to both the protein and the sugar molecule (Wolff et al., 1991, Harding and Beswick, 1988, Wolff and Dean, 1987, Thornalley et al., 1984, Hunt and Wolff, 1991). The second pathway is not completely independent from the first, as in this, the Amadori product (fructosamine) reacts with oxidative agents, like hydroxyl radicals, and undergoes modifications that lead to the formation of carboxymethyllysine (CML), one of the main AGEs (Ahmed et al., 1986, Booth et al., 1997, Thorpe and Baynes, 2003). The hydroxyl radicals can be produced either by transition metal-catalysed oxidation or by glucose autoxidation (Hunt and Wolff, 1991). This close interaction between glycation and oxidation leads to the development of the term glycoxidation, which describes the fact that AGEs are produced mostly from the interaction of these two processes (Baynes, 1991) (Figure 1.1).

Although indications and potential mechanisms are in place to suggest an active involvement of oxidative stress in protein glycation in normoglycaemia and hence the increase in the risk of chronic diseases, so far little evidence is available to support such a hypothesis.

1.5 Antiglycative capacity of antioxidants and polyphenols

In the search for compounds that can inhibit or slow the glycation reaction, antioxidants have attracted attention. The first AGE blocker identified is aminoguanidine (Brownlee et al., 1986); a dicarbonyl scavenging agent that reduces AGE production by removing the oxidatively produced precursors, like α-oxoaldehydes (Ahmed and Thornalley, 2002, Thornalley, 2003c). Aminoguanidine, like other glycation inhibiting compounds aspirin and ibuprofen, has the capacity to scavenge free radicals and improve redox status, which may contribute to their antiglycative capacity (Urios et al., 2007, Menzel and Reihsner, 1996, Thornalley, 2003c).

Lifestyle and diet can have a significant impact on glycation levels in normoglycaemia. Higher intake of vitamins C and E, and higher vegetable intake, have all been associated with lower levels of HbA1c in normoglycaemic subjects (Bates et al., 2004, Samaha et al., 2003, Sargeant et al., 2000, Vlassopoulos et al., 2013a, Boeing et al., 2000). Similarly polyphenol supplementation may lead to a reduction in HbA1c levels but the evidence is still inconclusive. Conversely, higher saturated fat intake and smoking have been related with higher HbA1c levels (Harding et al., 2001, Vlassopoulos et al., 2013a, Clair et al., 2011), while intakes of carbohydrates, protein, fibre and lower physical have no effect on glycation levels (Boeing et al., 2000, Sahyoun et al., 2005).

The antiglycative capacity of antioxidant vitamins and polyphenols has also been investigated, with *in-vitro* studies showing some polyphenols and phenolic acids to be even more effective than

aminoguanidine in inhibiting glycation (Wu et al., 2009, Choi et al., 2008, Kiho et al., 2004). Herb extracts and commonly consumed herbal preparations have been shown to inhibit glycation of albumin in experimental settings. Red wine, green tea, maté tea (Ilex paraguariensis) (Gugliucci et al., 2009, Bixby et al., 2005), cinnamon, garlic(Ahmad et al., 2007) and other herbs used to prepare hot drinks or added during cooking are rich in a variety of micronutrients with anti-glycative effects (Xi et al., 2008, Stote and Baer, 2008). A recent review of the literature by Xie et al. (2013) analysed results from 19 *in-vitro* trials and 11 animal studies and concluded that antiglycative capacity of polyphenols is linked to ring hydroxylation patterns. In this context, molecules with hydroxyl groups in the A and B rings (i.e. apigenin < luteolin, fisetin < quercetin, daidzein < genistein) those with multiple hydroxyl groups especially in the *ortho-* and *meta-* structure (i.e. sieboldin > phloridzin), the proanthocyanidin di/trimmers and the ellagitannins all showed increased antiglycative capacity. On the other hand, hydrogenation of the C2-C3 bond (i.e. eriodictyol < luteolin), methylation (i.e. diosmetin < luteolin) and the addition of rutinosides all decreased the antiglycative capacity (Xie and Chen, 2013).

The results of *in-vitro* studies were still heterogeneous and a thorough review of the glycation models and assays used would help to understand why translation of the findings to a physiological setting has not been forthcoming. Some of the reasons include use of high glucose or fructose concentrations, supraphysiological concentrations of polyphenols/phenolic acids and variability in the incubation period/temperature. Doses tested *in vitro* are, most of the times, supra-physiological, beyond concentration that could be reached via habitual consumption of phenolic-rich foodstuff. Most polyphenols are metabolised extensively by the gut microflora after ingestion due to their low bioavailability (Crozier et al., 2009, Del Rio et al., 2010), so studies focusing on the systemic effects of the parent compounds are likely to have low translational values. Phenolic acids, such as 3-hydroxyphenylacetic acid, 3,4-dihydroxyphenylacetic acid and caffeic acid on the other hand have a higher bioavailability than larger polyphenols and are more likely to exert systemic effects (Crozier et al., 2009, Del Rio et al., 2010).

Despite the extensive mechanistic evidence, epidemiological data on polyphenol consumptions are scarce. The main reasons include the difficulties and the biases associated with deriving polyphenol intake data from dietary records. The process involves the use of databases, such as PhenolExplorer (Rothwell et al., 2012) documenting the polyphenol content of foods (Rothwell et al., 2012, Bhagwat, 2013) and/or the analysis of Food Frequency Questionnaires (FFQ) to identify patterns of higher intake of polyphenol-rich foods. So far, there are no reports addressing the relationship between polyphenol intake and glycation levels. The reports associating polyphenol intake with diabetes risk have reached contradictory conclusions (Nettleton et al., 2006, Wedick et al., 2012, Song et al., 2005).

We performed a systematic review of the literature to identify all studies reporting findings relating antioxidant intake with protein glycation in normoglycaemia (Table 1). The literature research was carried out on PubMed and ISI Web of Knowledge. The search was inclusive of all years up to February 2014. The following search terms were used to identify relevant studies: phenol*, polyphenol*, elligitan, catechin, flavan*, flavon*, fruit*, vegetable*, spice*, tea*, cocoa or juice*, oil*, wine*, coffee, paired with treatment, intervention, consumption or supplementation. The wild-card term "*" was used to improve the sensitivity of the search by increasing the number of matches. The references of all relevant studies and reviews were hand-searched for additional studies. The review was limited to studies focusing on non-diabetic subjects.

Human trials with polyphenol rich supplements and foods are also limited and characterised by high heterogeneity and small sample sizes. In the past 20 years, only 14 trials used polyphenols as a means to reduce glycation in non-diabetic individuals, out of which two did not have any control group (Basu et al., 2013, Celec et al., 2013). Taken together, the results of these studies suggest that polyphenol supplementation fails to improve glycation markers in non-diabetic individuals. In populations with already established IGT, increased intake of polyphenols might be promising in reducing protein glycation (Cho et al., 2012, Fukino et al., 2008), but no hard conclusions can be made. Sample sizes ranged between 19-95 participants with different prevalence of overweight/obesity, hypertension, glucose abnormalities (metabolic syndrome, IGT) and age groups (Fukino et al., 2005, Banini et al., 2006, Fukino et al., 2008, Brown et al., 2009, Shiina et al., 2009, Aoki et al., 2012, Barth et al., 2012, Cho et al., 2012, Evans M., 2012, Vinson et al., 2012, Basu et al., 2013, Celec et al., 2013, Dallas et al., 2013, Miyazaki et al., 2013, Ogawa et al., 2013).

The bioactive molecules tested are also diverse, with flavonoids and catechins being the most common, but with dose administered ranging from 500mg to 2g daily. The main glycation marker measured is HbA1c but with study duration ranging from 2 to 12 weeks, it is questionable whether negative results are due to the limited duration (haemoglobin has a half-life of 12 weeks) or the supplement itself. Moreover, for the majority of studies, glycation was a secondary outcome and not the primary focus meaning that statistical power could be substantially affected. Furthermore, although the working hypothesis is that polyphenols will reduce glycation via improvements in the antioxidant capacity, there are no measurements to support that the interventions did anything towards improving the antioxidant status (beyond the post-prandial period).

Table 1.1 Supplementation trials reporting changes in glycation markers using antioxidant compounds/ antioxidant rich foods

Author	Study Population	Design	Treatment	Duration	Results
Davie et al. (1992)	Non-diabetics (n=12)	Parallel	Vitamin C (100mg) vs placebo	12 wks	1.13% decrease in GHb
					33% decrease glycated albumin
					16% increase in HbA1c with electrophoresis
Weykamp et al. (1995)	Non-diabetic (n=30)	Parallel	Vitamin C (0,750, 1500mg/d)	12 wks	No difference
Fukino et al. (2005)	Borderline diabetes (n=66)	Parallel	GTE capsule (458mg catechins; 106 mg caffeine) vs control	8 wks	No difference
Cheng et al. (2004)	Healthy (n=23)	Parallel	1000ug Cr vs placebo	24 wks	No changes reported
Banini et al. (2006)	Non-diabetic (n=23)	Parallel	Muscadine grape juice (150mL) vs control	4 wks	No effect on HbA1c
Camargo et al. (2006)	Healthy non diabetic (n=28)	Parallel	Vitamin C 1g/d or Vitamin E 400mg/d vs control	16 wks	No effect on HbA1c
Fukino et al. (2008)	Borderline diabetes (n=64)	Cross-over	GTE beverage (456mg catechins) vs control	8 wks	Reduction in HbA1c
Brown et al. (2009)	Overweight/obese (n=88)	Parallel	DGTE capsule (800mg EGCG)	8 wks	No effect
Shiina et al. (2009)	Healthy men (n=39)	Cross-over	Flavonoid-rich chocolate (550mg/d catechin,epicatechin and procyanidin) vs white chocolate	2 wks	No effect on HbA1c
Aoki et al. (2012)	Erectile dysfunction patients (n=23)	Parallel	10mg Pycnogenol, 115 mg L-arginine & 92mg aspartic acid/d vs placebo	8 wks	Moderate decrease in HbA1c (data not shown)
Barth et al. (2012)	Obese non diabetic (n=68)	Parallel	Cloudy Apple juice vs placebo	4 wks	No changes reported
			750mL/day (803 mg total phenol)		
Cho et al. (2012)	IGT (n=99)	Parallel	Sajabalssuk extract 3000mg/d vs placebo vs 1140mg/d pinitol (positive control)	9 wks	HbA1c reduction

Evans M. (2012)	Overweight/obese (n=19)	Parallel	Diabetinol 1050mg/d (dried fruit and orange peel supplement) vs placebo	12 wks	No difference
Vinson et al. (2012)	Hypertensive overweight (n=18)	Cross-over No wash-out	6-8 Purple majesty potatoes daily vs normal potatoes	4 wks	No difference
Basu et al. (2013)	Healthy WC<88/102cm (n=9)	Uncontrolled	POMx capsules (1506mg/d polyphenols)	4 wks	No reduction
Celec et al. (2013)	Young healthy (n=88)	Uncontrolled	2g/kg soybeans daily	1 wk	No effect on fructosamine/AGE fluorescence
Dallas et al. (2013)	Overweight healthy (n=95)	Parallel	900mg citrus fruit extract capsules (Sinetrol-XPur) vs placebo	12 wks	HbA1c increased in both groups, significantly smaller increase in Sinetrol group
Ogawa et al. (2013)	IGT (n=34)	Parallel multi-centre	1000mg Acacia polyphenol daily vs placebo	8 wks	No effect on HbA1c
Miyazaki et al. (2013)	Older healthy (n=52)	Parallel	631 mg Green Tea Catechins vs control 88.7 mg	14 wks	No effect on HbA1c

1.6 Hypothesis and research questions to be tested in this thesis

This thesis will be focused in studying the interactions of oxidative stress and molecules with antioxidant properties, particularly polyphenols and phenolic acids, with protein glycation. It was hypothesised that in normoglycaemic conditions, oxidative stress will be an important driver of the glycation process and that phenolic acids and polyphenols will exhibit an antiglycative capacity, both in an *in-vitro* physiologically relevant system and in an *in-vivo* system of increased oxidative stress.

So far experimental evidence support the active involvement of oxidative in the glycation process and epidemiological studies hint that glucose levels may not be the only driver of the process. When focusing in non-diabetic individuals conditions and lifestyle factors associated with increased oxidative stress can explain the variations in Hba1c levels.

RQ1: Is redox status associated with glycation in normoglycaemic individuals?

Given that glucose is tightly controlled, both in the fasting and post-prandial state, in individuals free of diabetes or impaired glucose tolerance there are still a gap in research to explain what drives the formation of fructosamine (HbA1c or glycated protein). Mechanistic studies so far have focused on the involvement of oxidative stress in the production of AGEs, either by fructosamine degradation or a-oxoaldehyde production. Little is known, though, on the impact of oxidative stress in the early step of the Maillard reactions. If so, should oxidative stress and glucose act synergistically or can prior oxidation of the protein molecule lead to increased glycation?

RQ2: Could oxidation promote fructosamine production in the presence of physiological glucose concentrations?

RQ2a: Are oxidised proteins more prone to glycation than the native forms?

As reviewed above, the plethora of studies employed to study the antiglycative capacity of polyphenols and phenolic acids are of low physiological values due to the study design. Even though this evidence is providing details on potential mechanisms of action and structures required for polyphenols to exhibit glycation protection there is a need to test their relevance in conditions simulating physiology.

RQ3: Can phenolic acids reduce glycation in a physiologically relevant *in-vitro* system?

Finally, if the hypothesis is that polyphenols/phenolic acids have the capacity to protect against glycation through oxidative stress regulation *in-vivo*, studies should be designed so to include participants with increased oxidative stress. This assumption is based on the fact that in a fairly

healthy population, the impact of any dietary intervention on markers of disease is likely to be subtle. Hence studies should either be aiming to recruit large samples to ensure statistical power for the detection of small effect sizes, or they should aim for high risk populations. When the working hypothesis is that polyphenol intake will be negatively correlated to glycation markers, through redox status regulation, individuals with increased oxidative stress (obese, pregnant, smokers) could be considered in high risk of glycative damage. Recruitment of such a population could allow for greater effect sizes between polyphenol intake and glycation markers to be detected allowing for a smaller sample size.

RQ4: Is polyphenol intake negatively correlated to glycation markers in a population at high risk of oxidative stress?

RQ4a: Is polyphenol intake and/or glycation related to maternal health or pregnancy complications?

RQ4b: Is maternal polyphenol intake and/or maternal glycation related to neonatal health?

Chapter 2: General Methods

This chapter describes the general methodologies chosen, developed and used throughout this thesis. The thesis comprises of 4 main experimental studies (chapters 3, 4, 5 and 6). Methods specific to each study are described in the relevant chapters. The objective of this thesis is to investigate the impact of oxidative stress and antioxidants on protein glycation in normoglycaemia. In an attempt to produce evidence of high translational value the thesis was designed to follow a mixed methods research designs.

2.1   Mixed methods research design

Mixed methods research is usually referred to research designs that combine qualitative and quantitative analysis in an attempt to improve the study design. Mixed methods research has been proposed as a superior research design that allows for pluralism and offers the best chance to approach a hypothesis from all the possible angles (Johnson and Onwuegbuzie, 2004). The combination of research methodologies from different fields is also common when studying the exposure to substances and its impact on health. Again in this case the combination of methodologies has been proposed as a tool to reach more biologically informative conclusions (Howard and Webster, 2013). When testing a new hypothesis, one is attempting to answer a currently unresolved issue, by a) generating a hypothesis for a mechanism of action/association and b) testing this hypothesis to be true. In order to generate valid hypotheses analysis of epidemiological data is a common first step. Following that the set-up of experimental studies could allow for these hypotheses to be tested and potential answers to be generated. The combination of epidemiology and experimentation in this case allows researchers to bring together data from various settings and analyse the recurrent patterns to reach biologically relevant and true conclusions (Schwarze et al., 2006).

In nutrition, discussing the totality of evidence available from epidemiology, *in-vitro* studies, animal studies and intervention trials when describing food-health interactions is not a new concept. This combined approach has been used in order to identify food patterns and food-borne agents that have the potential to modulate health, especially in the field of cancer (Wynder et al., 1994, Yun, 2003, Wolff et al., 1996, Hashim et al., 2005). In fact, the World Cancer Research Fund (WCRF) uses a similar approach when reviewing the evidence linking diet and cancer. Evidence from epidemiological studies, human interventions, animal studies and *in-vitro* studies are all reviewed together. The latter two studies are of particular importance as they provide with the evidence of 'plausible biological mechanisms', explaining the association proposed by the human trials and the epidemiology. Lack of investigations focusing on identifying those biological

mechanisms is considered a substantial drawback and the evidence is unlikely to be characterised as being convincing, probable or even suggestive (Wiseman, 2008).

This thesis follows a design similar to the epidemiological approach in investigating disease problems as proposed by the Food and Agriculture Organisation (FAO) (Putt et al., 1987). In this model there are 8 stages in each investigation, the diagnostic, the descriptive, the investigative, the experimental, the analytical, the intervention, the decision-making and the monitoring phase. Once a disease/health problem is confirmed (diagnostic phase), an analysis of the population at risk/of interest is performed to generate hypotheses about the determinants (descriptive phase). In the investigative phase, these hypotheses are initially tested with a series of field studies which describe the exposures and risk factors in more detail. If the hypotheses are promising, then experiments are performed to test the capacity of single-agent exposures to explain the disease/outcome (analytical stage). By the analysis of the results of the experiments, a set of agents capable to modulate the outcome are identified (analytical phase). Following that, interventions aiming to manipulate the exposures of interest take place and their effect on disease outcome is measured (intervention phase). At this point, the research phase of the investigation is nearly complete. The next two phases, the decision-making and monitoring phase, see the interventions being applied in the field and monitor their long-term outcomes.

In this thesis, epidemiological data were used to generate and test hypotheses in a population level, followed by a series of *in-vitro* experiments that investigated the molecular mechanisms in place to explain the previous findings. The biological relevance of these mechanisms was then tested using samples from a nutritional intervention (randomised controlled trial).

2.2   Epidemiological data

The Scottish Health Survey (SHS) is a population wide investigation aimed to provide with a detailed picture of health, health related behaviour and their changes over time in Scottish households. The Survey started in 1995 and was repeated in 1998 and 2003. In 2008 the SHS design was changed and it is now conducted annually until 2015 (Scottish Government, 2014). The SHS aims to estimate the prevalence of health conditions and to identify and monitor related disease risk factors over time, making sure that all regions and population subgroups are successfully represented in the time of sampling. To achieve that a multi-stage stratified probability sampling of eligible households is performed (Scottish Government, 2014).

The survey takes place as a two-stage interview process. During the personal interview a trained interviewer collects data on health, disability, health service use, cardiovascular and respiratory disease, smoking, drinking, common health problems and eating patterns/physical activity. The second nurse visit is focused on obtain blood, urine and saliva samples, anthropometrical measurements and use of drugs (prescribed) and vitamins. From 1995 to 2003 all the sample was offered both visits but from 2008-2011 only a sixth of the sample was offered the nurse visit. The SHS has a strong focus on nutrition with fruit and vegetable consumption been measured annually as part of the core content, alongside smoking, drinking and physical activity and a more elaborate investigation of eating habits taking place biennially (Scottish Government, 2013). As such the SHS can be a useful tool in nutritional epidemiological research, allowing the study of association between biochemical markers, nutrition and health.

2.3   *In-vitro* design

The study of the glycation mechanisms has been utilising *in-vitro* protein model systems. The most commonly used proteins are LDL, albumin and collagen (Li et al., 1996, Xi et al., 2008, Vinson and Howard, 1996, Harris et al., 2014, Kazeem et al., 2012, Xie and Chen, 2013). Albumin the most abundant protein in circulation has a half-life of 14-28 days and is a known glycation target (Schultze and Heremans, 1966, Schleicher et al., 1993). In fact measurement of albumin glycation as fructosamine has been part of clinical practice, diabetes risk monitoring and it may still be a useful marker for individuals with altered red blood cell turnover (Inaba et al., 2007, Takahashi et al., 2007, Peacock et al., 2008, Koga and Kasayama, 2010, Selvin et al., 2014). The study of glycation promoters and inhibitors has not been yet standardised with different groups employing different settings, in terms of duration, protein concentration and sugar concentration (Li et al., 1996, Xi et al., 2008, Vinson and Howard, 1996, Harris et al., 2014, Kazeem et al., 2012, Xie and Chen, 2013, Joglekar et al., 2013). Also although glucose is the most common circulating sugar, some reports have used fructose and ribose as glycation drivers (Sompong et al., 2013, Kontogianni et al., 2013); others have also used a-oxoaldehydes, like methylglyoxal (Tarwadi and Agte, 2011, Li et al., 2008, Gutierrez, 2012).

Although from a mechanistic point of view these studies may be of importance, their biological relevance is limited. Only a small number of studies have attempted to replicate the physiological process of glycation *in-vitro* by using concentrations and duration relevant to physiology. In this thesis the *in-vitro* setting was designed to mimic physiology as much as possible. The glycation experiment lasted for 28 days (4 weeks) with measurements of glycation taking place at both 14

days (2 weeks) and 28 days (4 weeks). Albumin concentration was chosen to be within the physiological 35-50g/L and glucose levels ranged from 5mM to 30mM. The choice of glucose levels was such that it would allow for the investigation to focus both in normoglycaemia (5mM glucose) and diabetes (10mM glucose) (American Diabetes, 2000). Glucose concentrations of 20 and 30mM glucose were chosen to test the same hypothesis in glucose concentrations previously used in the literature to allow for the results of the experiments to be compared with previous reports (Voziyan et al., 2003, Chesne et al., 2006, Bourdon et al., 1999).

Similarly the concentrations of all the reagents added in the incubation mix to act as promoters or inhibitors of glycation were physiologically relevant. Reviews of the literature were performed prior to any experimental set-up to inform the choice of the agents of interest, their potential synergistic/antagonistic effect and their concentration in human plasma. Details of these reviews are presented in the following chapters where appropriate and relevant.

2.4   Experimental data from randomised controlled trials

As mentioned earlier, the study of the association between redox status and glycation may be more sensitive if the population at focus is at high risk of oxidative stress. Epidemiological investigations have showed that obesity and smoking are associated with impaired redox status (Vargas et al., 2013, Pou et al., 2007, Isik et al., 2007, Marangon et al., 1998). Similarly during pregnancy there is a significant increase of oxidative stress and decrease in antioxidant capacity, especially during the 3rd trimester (Hung et al., 2010, Toescu et al., 2002). Obesity during pregnancy has been associated with increased risk of pre-eclampsia, gestational diabetes and other complications (Ramachenderan et al., 2008, Baeten et al., 2001, O'Brien et al., 2003). At the same time oxidative stress and changes in the level of AGEs and sRAGE have also been shown to carry some predictive value of pregnancy-complications (Raijmakers et al., 2004, Sharma et al., 2006, Moretti et al., 2004, Harsem et al., 2008, Naruse et al., 2012), giving room to hypothesise that obesity during pregnancy may be related with a deregulation of the oxidation and glycation pathways.

All the above suggest that obese pregnant women are likely to serve as a population in high risk of oxidative in which antioxidant/polyphenol intake could deliver multiple benefits including glycation reduction. Furthermore, with increasing indications for the involvement of glycation in pregnancy complications and fetal outcomes, such a study will provide with useful information about the potential impact of diet on maternal and neonatal health.

For the purpose of this thesis we analysed samples and data from the UK Pregnancies Better Eating and Activity Trial (UPBEAT) pilot, a multi-centre UK trial with a randomised controlled

design. The detailed designed of the trial has been published elsewhere (Poston et al., 2013, Briley et al., 2011) and discussed in more detail in the relevant chapter. Participants in the UPBEAT trial were offered an 8-week intervention aiming to reduce the dietary glycaemic index (GI) and increase physical activity or standard care. The reduction in glycaemic index was to be achieved by higher consumption of low glycaemic index foods and replacement of sugar sweetened beverages with low GI alternatives. Alongside, GI reduction women in the intervention arm were asked to replace saturated fats with mono and polyunsaturated fats (MUFA, PUFA). As low glycaemic food products like wholemeal cereal, vegetables and legumes are also high in antioxidant compounds (Ludwig, 2002, Slavin, 2003, Hu, 2003, Jenkins et al., 2002) we hypothesised that women in the intervention arm could benefit from an improved redox status compared to the control.

2.5   Statistical analysis

Detailed presentations of the statistical analysis performed for each experiment is presented in relevant chapters.

Generally, epidemiological data and data from human trials were initially tested for normality using the Kolmogorov-Smirnoff test. Data were then presented as mean and standard deviation (SD) or median and interquartile range (IQR) as appropriate. No transformation of the data was performed to force them in following a normal distribution. Differences in means/medians were assessed using the Student's t-test or ANOVA or the non-parametric equivalents. When *post hoc* comparisons were applied in ANOVA tests, the Tukey *post hoc* test was applied. In the case of epidemiological data all analyses were performed using parametric tests following the central limit theorem. The central limit theorem suggests that given a large enough population size normality of the data can be assumed and hence parametric tests and regression can be performed without prior transformation, as supported by the work of (Lumley et al., 2002).

Regression analysis (linear and logistic) was employed to investigate potential relationships between exposures (oxidative stress, antioxidant intake etc.) and outcomes of interest (e.g. glycation levels). All regression analyses were performed initially as a non-adjusted model and then as a fully adjusted model. In the fully adjusted model all the known confounders of the relationship of interest were added in the model without previous stepwise regression being performed.

In the *in-vitro* studies all incubations were performed in 5 or 6 replicates to ensure sufficient numbers to be entered in the statistical analysis. All data were again assumed to be normally

distributed and so Student's t-test or ANOVA were employed to test for differences among mean values. All data were presented as mean values with standard deviation. When *post hoc* comparisons were applied in ANOVA tests, the Tukey post-hoc test was applied, in this case as well. In order to test the interaction between two different exposures (i.e. oxidative stress and glucose concentration) a two-way ANOVA model was applied to the data, again with Tukey post-hoc correction, if needed.

Statistical significance was set at a p-value <0.05 for two-tailed comparisons. All tests were performed using the PASW 18 statistical software.

Chapter 3: Influence of smoking and diet on glycated haemoglobin and 'pre-diabetes' categorisation: a cross-sectional analysis

This chapter is presented exactly as published in BMC Public Health

Abstract

Background: The new HbA1c criteria for diagnosis of pre-diabetes have been criticised for misdiagnosis. It is possible that some elevation of HbA1c is not driven by hyperglycaemia. This study assesses associations of HbA1c, commonly assumed to relate solely to glucose concentration, with (i) smoking, a major source of ROS and (ii) fruit & vegetables consumption associated with improved redox status.

Methods: One-way ANOVA, Chi-squared and multivariate linear regressions, adjusted for all known confounders were used to explore associations of HbA1c with self-reported smoking status and fruit & vegetables consumptions in the Scottish Health Surveys 2003-2010, among individuals without known diabetes and HbA1c<6.5%.

Results: Compared to non-smokers (n=2831), smokers (n=1457) were younger, consumed less fruit & vegetables, had lower physical activity levels, lower BMI, higher HbA1c and CRP (p<0.05). HbA1c was higher in smokers by 0.25 SDs (0.08%), and 0.38 SDs higher (0.14%) in heavy smokers (>20cigarettes/day) than non-smokers (p<0.001 both). Smokers were twice as likely to have HbA1c in the 'pre-diabetic' range (5.7 - 6.4%) (p<0.001, adj.model). Pre-diabetes and low grade inflammation did not affect the associations. For every extra 80g vegetable portion consumed, HbA1c was 0.03 SDs (0.01%) lower (p=0.02), but fruit consumption did not impact on HbA1c, within the low range of consumptions in this population.

Conclusion: This study adds evidence for to relate smoking (an oxidative stress proxy) to protein glycation in normoglycaemic subjects, with implications for individuals exposed to ROS and for epidemiological interpretation of HbA1c.

3.1 Background

Patients with large waists and related, potentially-reversible, metabolic features are at risk of developing type 2 diabetes and a range of chronic diseases (Han et al., 2006). Their specific pathologies are characterised by pro-inflammatory states and a shift in tissue redox homeostasis towards excess free-radical activity (Hermsdorff et al., 2011). Redox status is influenced by diet and lifestyle factors, including fruit and vegetable consumption, cigarette smoking and by inflammatory disease activity (Alberg, 2002, Moriarty et al., 2003, Lesgards et al., 2002). Smoking is a well-established risk factor for diabetes, as shown by a recent meta-analysis (Willi et al., 2007), contributes to the production of ROS and increases production of inflammatory molecules, β-cell dysfunction and end-organ protein damage (Fagard and Nilsson, 2009, Naudi et al., 2012, Cerami et al., 1997). On the other hand, low fruit and vegetable consumption is associated with impaired redox status in young, healthy populations (Lesgards et al., 2002) and diets rich in fruit and vegetable with lower concentrations of oxidative stress markers (Esposito et al., 2011, Dai et al., 2008). Individuals exposed to higher levels of oxidative stress experience greater benefits in their redox status from fruit and vegetables consumption (Peluso et al., 2012).

Protein glycation is a common form of protein damage, and approximately 1%-16% of albumin is glycated in normoglycaemic blood (Shaklai et al., 1984, Selvin et al., 2011), which has been associated with metabolic deterioration. Advanced Glycation Endproducts (AGEs) are involved in the aetiology of various chronic disease (Yan et al., 2010), especially diabetes and its vascular complications. Protein glycation levels are determined by the concentration of sugars (mainly glucose) (Goldstein et al., 2004, Nathan et al., 2008) and dependant on the protein half-life, as well as fructosamine removal through the action of fructosamine-3-phosphokinase (Delpierre et al., 2002). An individual's pro-oxidant status, however, has been speculated to be involved in later stages of the glycation reaction, leading to the formation of glycoxidation products, like pentosidine (Fu et al., 1994, Baynes, 1991). Moreover, glucose autoxidation, associated with increased oxidative stress, may play a role in promoting Maillard product formation, in the early glycation stages (Chetyrkin et al., 2011). HbA1c is an early glycation product used diagnostically as a specific marker for glucose exposure. HbA_{1c} has become established for monitoring of glycaemic status in diabetes, as an indicator of glucose levels in the previous 90 days, and more recently its diagnosis (ADA, 2011). HbA_{1c} relates strongly to tissue damage in diabetic patients and it has also been found to predict coronary heart disease (CHD) and cancer in non-diabetic individuals, even within the 'normal' non-diabetic range (4.9 - 6.3%) (Brewer et al., 2008). The concentration of HbA_{1c} is usually assumed to relate mainly to glucose concentration in populations with similar red blood cell turn-over. Since blood glucose fluctuations are minimal within HbA1c levels <5.7%, it

seems possible that the relatively large variations on HbA$_{1c}$ concentration might reflect variations in redox status, and could indicate wider protein glycation. Supporting this concept, we have shown previously, in national survey data, that HbA1c in non-diabetic subjects, correlates with CHD risk factors, but that it is inversely correlated with dietary intake of fruits and vegetables, and with both dietary intake and plasma concentration of dietary antioxidants (Vitamin C, Vitamin E, Vitamin B6) (Bates et al., 2004).

Since the process of glycation is non-enzymatic, it is relatively slow, so day-to-day variations are unlikely to have a major impact. This is an advantage for health surveys and screening. The Scottish Health Survey (SHS) comprises representative population-based surveys, in a population at high risk of CHD (Government, 2008), conducted every 3-5 years from 1995 until 2003, and annually since 2008. We have investigated whether lifestyle drivers of redox status (cigarette smoking, fruit and vegetable consumption) are associated with protein glycation, using HbA1c as a marker of the process, in sub-samples of non-diabetic adults.

3.2 Methods

This study is a secondary analysis of the anonymised data from the Scottish Health Survey and the Health Survey for England. Original ethical approval for the Scottish Health Surveys was granted by the Multicentre Research Ethics Committee (Scotland). As the data are in the public domain and available through the Economic and Social Data Service (ESDS), this study required no additional ethical approval.

Subjects

Data from the 2003, 2008, 2009, and 2010 SHSs were compiled in order to create the large database used in the current analysis. The SHS is a cross-sectional nationally representative survey reporting the health and health-related behaviour of people living in private households in Scotland, using a multistage, stratified, clustered probability-sampling design. Full survey procedures are described elsewhere (Lynch et al., 1996, Bromley C, Bromley C); a brief summary is given here.

General methods

During two household visits, data on demographic, economic, occupational, age, sex, general health and health related habits were collected. Weight was measured to the nearest 100g using electronic scales. Height was measured to the nearest millimetre using a stadiometer. Body Mass Index (BMI) was calculated as weight (kg) divided by height squared (m^2). The waist was measured at the midpoint between the lowest rib and upper margin of the iliac crest. The measurements were recorded to the nearest millimetre with at least two measurements within 5cm combined to provide a mean (Lean et al., 1995).

Dietary measures

Two different tools were used to assess eating habits in the Scottish Health Surveys. One, a FFQ validated against weighed intakes (Lean et al., 2003), was specifically designed to assess fruit and vegetable intake. Responders were asked about the total number of portions of vegetables (fresh, frozen or canned) and vegetables in composites, salads, pulses, fruit (fresh, frozen or canned), dried fruit and fruit in composites consumed in the 24h preceding the interview. From the available nutritional information, variables that reported total portions of vegetable, total portions of fruit and the two of them combined were selected for the current analysis.

Smoking habits

Participants were asked to report if they were currently smoking, ex-occasional or ex-regular smokers, or never smoked. Present smoking status was classified as light (under 10 cigarettes a day), moderate (10 to under 20 cigarettes a day), heavy (20 or more cigarettes a day) and non-smoker.

Physical activity

Frequency of participation was assessed for various domains of activity, including leisure time sports and exercise (for at least 15 minutes per occasion). The total physical activity was then categorised to 'low/medium/high' according to the metabolic equivalents spent during each activity and the total activities reported.

Biochemical and other measurements

Serum CRP was measured by N Latex high sensitivity mono-immunoassay on the Behring Nephelometer II analyzer (coefficient of variation <6%, limit of detection 0.17mg/l) in nmol/L (conversion factor to mg/L= 0.105) and total glycated haemoglobin (HbA1c) analysis was carried out in the Biochemistry Department at the RVI using the Tosoh G7 HPLC analyser, which was calibrated using Diabetes Control and Complications Trial (DCCT) standards with coefficient of variation <2.5%.

Statistical analysis

In order to exclude undiagnosed diabetes cases, the cut-off point of HbA_{1c} ≥6.5% (48mmol/mol) was employed (ADA, 2011). Participants without diagnosed diabetes and with HbA_{1c} ≤6.5% (48mmol/mol) were included in the analysis. Pregnant women were excluded. Individuals with HbA1c between 5.7% (39mmol/mol) and 6.4% (46mmol/mol) were classified as being at high risk or 'pre-diabetic' (ADA, 2011). Body mass index, mean waist circumference, age and CRP levels were used as continuous variables. CRP was also used in order to identify and exclude individuals with low grade inflammation (CRP >28.5nmol/L or >3mg/L) (Wildman et al., 2008, Grundy et al., 2004). Data were checked for normality and homoscedasticity using the Kolmogorov-Smirnoff test, and for skewness. Parametric tests were used for data with normal and non-normal distributions since, according to the Central Limit Theorem (Lumley et al., 2002), parametric tests can safely be used with skewed data when the sample size is over 500. The Student's t-test, one way-ANOVA and χ^2 test were used to examine the differences among groups of smoking status and fruit and vegetables intake in HbA_{1c} levels. General linear models were used to compare HbA_{1c} levels among smoking groups that were adjusted for age and sex. Multivariate linear regression was used to describe the effect of smoking and fruit and vegetables intake on HbA_{1c}, after adjusting for age, sex, ethnic group, socioeconomic group, activity levels, BMI, waist circumference, CRP levels, vitamin supplementation and year of survey. Logistic regression was performed to investigate the association of smoking status and presence of increased risk for diabetes. In the case of fruit and vegetable intake the model was adjusted to include smoking in the confounders. All analyses were performed using PASW Statistics (18.0.0) and statistical significance was taken as p<0.05.

3.3 Results

Glycated haemoglobin levels (HbA$_{1c}$) were higher in ex-smokers and current smokers compared to non-smokers (Table 3.1) and this increasing trend (p<0.001) was seen even when the age-sex adjusted means were plotted against smoking status (Figure3.1). The trend remained consistent when the ex-smokers were split into those who smoked occasionally and those who smoked regularly (Figure 3.1). The same analysis of age-sex adjusted HbA1c levels was conducted among groups based on number of cigarettes smoked per day, with similar results (p<0.001) (Figure3.2).

Figure 3.1 Age-sex adjusted mean (SD) of %HbA1c according to smoking status
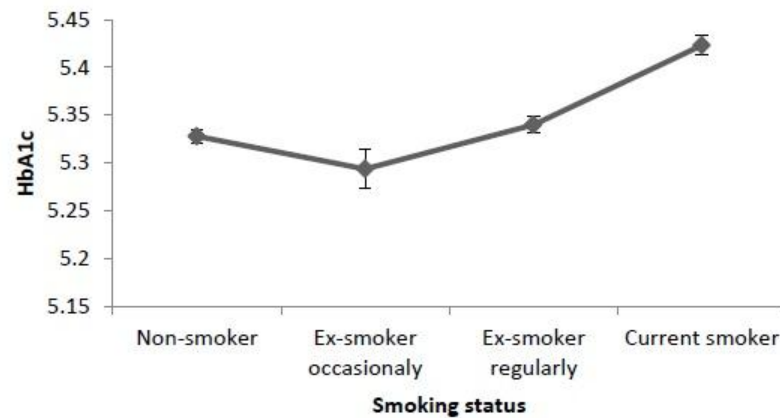
Figure 3.2 Age-sex adjusted mean (SD) of %HbA1c according to number of cigarettes/day
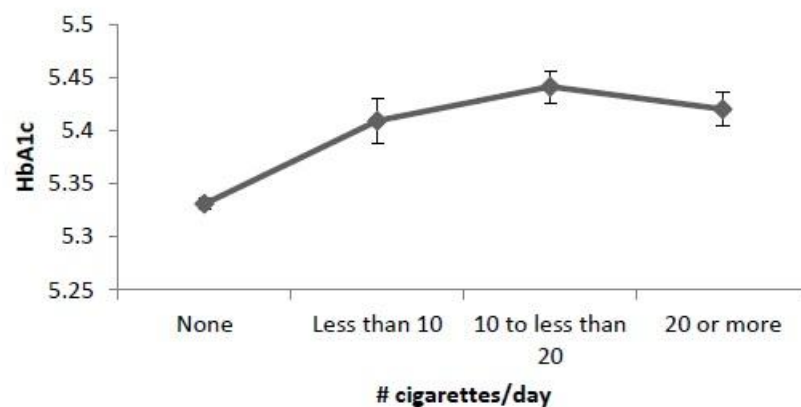
Table 3.1 Descriptive characteristics of the population in total and according to smoking status

		Smoking status		
	All	**Non-smokers**	**Ex-Smokers**	**Current Smokers**
	(n=6120)	(n=2831)	(n=1832)	(n=1457)
Age (years)	50.5±16.9 (18.0-95.0)	49.0±17.2 (18-95)	56.2±16.0 (19-94)*	46.1±15.3 (18-91)*
Sex (% Male)	45.2	42.6	49.0*	45.4*
Social class (% High[†])	43.2	50.6	44.2*	27.7*
Body Mass Index (kg/m^2)	27.3±4.8 (13.3-62.7)	27.3±4.8 (15.2-53.3)	27.8±4.5 (13.3-54.5)*	26.4±5.1 (15.1-62.7)*
Waist circumference (mm)	91.6±13.2 (54-152)	90.5±13.2 (54-152)	93.8±13.0 (60-152)*	89.8±13.4 (58.5-147)
Physical activity (%Low)	30.7	28.1	33.7*	32.1*
HbA1c (%)	5.4±0.4 (2.8-6.4)	5.3±0.4 (2.8-6.4)	5.4±0.4 (3.8-6.4)*	5.4±0.4 (2.9-6.4)*
(mmol/mol)	*36±4.4 (30.6-46)*	*34±4.4 (30.6-46)*	*36±4.4 (18-46)*	*36±4.4 (31.7-46)*
CRP (nmol/L)	33.3±65.7 (1.9-971)	27.6±55.2 (1.9-971)	36.2±75.2 (1.9-95.4)*	40.9±68.6 (1.9-762)*
Total fruit portions/d	2.0±1.8 (0.0-20.0)	2.3±1.8 (0.0-12.5)	2.2±1.8 (0.0-10.5)	1.3±1.7 (0.0-22.0)*
Total vegetable portions/d	1.3±1.4 (0.0-22.0)	1.4±1.3 (0.0-20.0)	1.4±1.3 (0.0-15.3)	1.1±1.3 (0.0-17.7)*
Total fruit & vegetable portions/d	3.4±2.5 (0.0-31.4)	3.7±2.4 (0.0-22.5)	3.6±2.4 (0.0-25.3)	2.5±2.4 (0.0-31.4)*

Data presented as mean ± SD (range) [†]High social class: Professional and managerial technical;* compared to non-smokers; $p < 0.05$

Current smokers were twice as likely as non-smokers to have HbA1c in the pre-diabetes range (Table 3.2). Lighter smokers (<10 cigarettes/day) were almost twice as likely as non-smokers to have HbA$_{1c}$ in the pre-diabetes range (OR 1.88), while smoking 10 to 20 cigarettes per day increased the risk for HbA1c ≥5.7% (39mmol/mol) more than 2-fold (OR 2.63) (Table3.2). Being an ex-occasional or ex-regular smoker was not associated with higher chances of being classified in the high risk/pre-diabetes category of HbA1c.

Body mass index (BMI) differed significantly by smoking status: current smokers had a lower BMI than non-smokers when ex-smokers had a significantly higher BMI than non-smokers (Table 3.1). Smoking is associated with poorer social circumstances, while ex-smoking status is more frequent among those with better social circumstances (% high social class 44.2 vs. 27.7 in ex-smokers and current smokers respectively p<0.001) (Table 3.1). Current smokers included younger subjects than non-smokers (46.1±15.3 vs. 49.0±17.2 years; p<0.001), whereas ex-smokers included older subjects. There was no difference in waist circumference between current smokers and non-smokers, but ex-smokers had a higher mean waist circumference than non-smokers (p<0.05). Current smokers consumed fewer portions of fruit, vegetables, and the two combined, than non-smokers and a higher proportion were classified as having low physical activity. Although ex-smokers also included more subjects classified as having low physical activity than non-smokers, they were no different for fruit and vegetable consumption. CRP concentrations were greater among smokers, both ex-smokers and current smokers having higher values than non-smokers (Table 3.1). Ex-smokers and current smokers had CRP levels significantly higher than the 28.5nmol/L cut-off indicating high risk for metabolic diseases range and low-grade inflammation (p<0.001; data not shown). In all following analysis the above mentioned variables were used as confounders.

Consumption of vegetables was associated with having marginally lower chances of having HbA$_{1c}$ in the pre-diabetes range. This effect remained after adjustment for various confounders, using either smoking status or number of cigarettes per day (Table 3.2).

Multivariate linear regression showed that current smokers have higher HbA1c than non-smokers, by 0.08% (0.9mmol/mol) (equal to 0.25 the SD of this population). Heavy smoking (20 or more cigarettes a day) is associated with a larger effect of 0.14% (1.5mmol/mol) (equal to 0.38 the SD of this population) greater HbA1c. The regression model explained ≈35% of the variance in HbA$_{1c}$. From all the confounding factors employed in this study only year of survey had a significant effect on HbA1c levels in the full factorial model (coefficient 0.13±0.04, p<0.001) (data not shown). HbA1c levels were lower by 0.01% (0.1mmol/mol) for each extra portion of vegetable

consumed, after controlling for number of cigarettes per day. The same was not found for fruit portions per day or fruit & vegetables portion combined (Table 3.3).

When individuals with HbA1c in the pre-diabetes range were assessed alone, the R^2 value of the multiple regression model for the narrow range of HbA1c was only 0.06, leaving a large proportion of variance unexplained (data not shown). Thus neither vegetable nor fruit consumption had an effect on HbA1c (Table 3.3). There was no interaction between smoking status and HbA1c level (p=0.20) but there was weak evidence for an association between number of cigarettes per day and greater HbA1c levels (p=0.06).

In order to avoid confounding effects from any mild metabolic disruptions associated with the pre-diabetic status or low-grade inflammation, the above analysis was conducted among individuals with HbA1c below the 5.7% (39mmol/mol) cut-off, and with a CRP lower than 28.5nmol/L. Heavy smoking (10-20 cigarettes/day) was still associated with ≈0.1% (1.1mmol/mol) (equal to 0.28 the SD for this population) greater HbA1c than non-smoking (Table 3.3). Although vegetable consumption was significantly (inversely) associated with HbA1c among the non-diabetic individuals as a whole, the level of significance dropped for this analysis restricted to individuals with HbA1c less than 5.7% (39mmol/mol) (p=0.09). However, individuals with both HbA1c <5.7% (39mmol/mol) and also CRP<3mmol/L had significantly lower HbA1c, by 0.01% (0.1mmol/mol) (equal to 0.03 the SD of this population) (p=0.02) for every extra portion of vegetables consumed (Table 3.3).

Table 3.2 Logistic regression for smoking status and fruit & vegetable consumption predicting high risk of diabetes

	OR	95%CI	p-value
Smoking status			
Non-smoker (contrast)			
Ex-occasional	0.88	0.59-1.32	0.54
Ex-regular	1.11	0.92-1.34	0.26
Current smoker	*2.25*	*1.84-2.75*	*<0.001*
# cigarettes/day			
None (contrast)			
Less than 10	*1.88*	*1.31-2.71*	*<0.001*
10 to less than 20	*2.63*	*2.04-3.39*	*<0.001*
More than 20	*2.06*	*1.57-2.71*	*<0.001*
(adjusted for smoking status)			
Vegetable intake	*0.94*	*0.89-1.01*	*0.08*
Fruit intake	0.99	0.94-1.03	0.56
Fruit & vegetable intake	0.98	0.95-1.01	0.17
(adjusted for # cigarettes/day)			
Vegetable intake	*0.95*	*0.89-1.01*	*0.09*
Fruit intake	0.99	0.94-1.03	0.53
Fruit & vegetable intake	0.98	0.95-1.01	0.17

*adjusted for age, sex, ethnic group, social class, physical activity level, CRP, BMI, waist circumference & year of study

Table 3.3 Regression analysis summary for smoking status and fruit &vegetable consumption with %HbA1c levels

a) Non-diabetics (HbA1c<6.5%) (n=5425)	Coef*	SE	p-value
Smoking status	*0.027*	*0.004*	*<0.001*
# cigarettes/day	*0.047*	*0.005*	*<0.001*
Vegetable intake[†]	*-0.009*	*0.004*	*0.008*
Fruit intake[†]	0.001	0.003	0.754
Fruit & vegetable intake[†]	-0.002	0.002	0.236
b) High risk of diabetes (HbA1c 5.7%-6.4) (n=1391)			
Smoking status	0.050	0.004	0.201
# cigarettes/day	*0.010*	*0.005*	*0.060*
Vegetable intake[†]	0.001	0.004	0.947
Fruit intake[†]	0.004	0.003	0.170
Fruit & vegetable intake[†]	0.002	0.002	0.292
c) Low risk of diabetes (HbA1c<5.7%) (n=4155)			
Smoking status	*0.017*	*0.003*	*<0.001*
# cigarettes/day	*0.031*	*0.005*	*<0.001*
Vegetable intake[†]	-0.005	0.003	0.092
Fruit intake[†]	0.001	0.002	0.603
Fruit & vegetable intake[†]	-0.001	0.002	0.605
c) Low risk of diabetes(HbA1c<5.7%) & CRP<28.5nmol/L (n=3172)			
Smoking status	*0.014*	*0.004*	*<0.001*
# cigarettes/day	*0.029*	*0.005*	*<0.001*
Vegetable intake[†]	*-0.009*	*0.004*	*0.021*
Fruit intake[†]	0.002	0.003	0.546
Fruit & vegetable intake[†]	-0.001	0.002	0.464

*adjusted for age, sex, ethnic group, social class, physical activity level, CRP, BMI, waist circumference & year of study; [†]adjusted for #cigarettes/day

3.4 Discussion

HbA1c is commonly regarded mainly as a biomarker for blood glucose levels; however, observing the substantial variation in HbA1c among non-diabetic individuals, unlikely to result from sustained differences in blood glucose, we postulated that HbA1c may also reflect oxidative stress or redox status. In our analysis, we have demonstrated that smoking (a proxy for oxidative stress) is positively associated with protein glycation, as measured by HbA1c levels in non-diabetic subjects. This finding adds complexity to the evidence that smoking increases the risk of developing type 2 diabetes (Alter et al., 2012, Willi et al., 2007).

Although smoking has been extensively studied as a risk factor for deteriorating diabetic status and as promoting end-organ damage in diabetic subjects, evidence on the effect of smoking on protein glycation and diabetes progression is not so clear. A large epidemiological study conducted in the USA has found similar results with smokers having a relative 7% increase in HbA1c levels compared to non-smokers, in a population free of diabetes (Clair et al., 2011). An early report of 191 hypertensive and normotensive non-diabetic subjects detected a statistically significant difference in HbA1c levels between smokers and non-smokers (Nilsson et al., 1995). The same was reported in a sample of 3,240 healthy non-diabetic adults (Simon et al., 1989) and in 1,773 middle-aged non-diabetic participants from the Potsdam cohort of EPIC (Boeing et al., 2000) where smoking was associated with increased HbA_{1c} levels in both sexes. A recent Dutch study assessing the role of increased waist circumference (>88cm for women, >102cm for men) on protein glycation, measured by skin auto-fluorescence, in non-diabetic individuals identified current smoking as an important confounder in the association, which agrees with our data (den Engelsen et al., 2012). Among these diverse studies, some can be considered valid, but others have low numbers and weaknesses in design. The current study benefits from the large sample size and the numerous diabetes related variables available, in order to control for confounding.

In the current study we have also shown that smoking remains positively correlated with HbA1c in a subsample of participants who are not affected by any metabolic derangements from pre-diabetes (with HbA1c<5.7%, 39mmol/mol) and in a subsample with both HbA1c<5.7% (39mmol/mol) and CRP levels <28.5nmol/L to exclude any influence from low-grade inflammation. This fractioning of the population was performed on the basis that individuals with HbA_{1c} levels within the prediabetic range, would already have some degree of abnormalities in glucose metabolism. These abnormalities are most likely to explain the variation in HbA1c levels in this population subgroup (Monnier et al., 2003, Bastyr et al., 2000). These results provide robust evidence for a true association between oxidative stress and protein glycation. In fact, we did not find evidence for the same association with smoking among the individuals with HbA1c in

the pre-diabetic range 5.7 - 6.4% (39 - 46mmol/mol), probably because of smaller number of participants in this group, with a range of HbA_{1c} too narrow to detect an influence, however the main driver protein glycation in this group is likely to be their altered glycaemia and glucose level fluctuations. It seems likely that oxidative stress and redox status are more potent drivers of protein glycation on a background of normal glucose metabolism, but elevated blood glucose concentrations in the fasting and/or post-prandial states would dominate. Unfortunately lack of data on blood glucose levels did not allow for this association to be investigated in the present study. Measuring both HbA_{1c} and glucose concentrations in large epidemiological studies, together with indicators of exposure to oxidative stress and antioxidant/anti-inflammatory influences, would be of great value to discriminate these effects.

There was some evidence in the present study that vegetable consumption (a proxy for antioxidant influences on redox status) was inversely associated with HbA_{1c} levels in all non-diabetic subjects (HbA1c <6.5%, 48mmol/mol) and among individuals with low HbA1c (<5.7%, 39mmol/mol) and low CRP (<28.5nmol/L), suggesting some benefit from diets high in antioxidant rich sources. It is likely that fruit would have the same physiological effect, but the present study, in a population notorious for its low fruit consumption (and a narrow range of fruit consumptions for statistical analysis) was unable to detect an effect. Our data have shown mean intakes of vegetables to be 1.3±1.4 portion per day and fruit 2.0±1.8 portions per day.

In a large study of older English adults, by Bates et al (Bates et al., 2004), both dietary antioxidant consumption and antioxidant plasma levels of non-diabetic individuals were found to have inverse associations with HbA1c levels. There was also an inverse correlation between plasma vitamin C concentration and HbA1c. A similar inverse relationship has been found for vitamin C and vitamin E in a younger population, also suggesting that antioxidant intake and hence antioxidant capacity and redox status are playing a role in protein glycation in normoglycaemia (Boeing et al., 2000). A link between antioxidant intake and protection against protein glycation is supported by *in-vitro* evidence clearly showing that antioxidants, including vitamin C, flavonoids and tocopherols reduce glycation of proteins (Davie et al., 1992, Krone and Ely, 2004), and pro-oxidants, such as H_2O_2 and lipid peroxides, drive glycation of haemoglobin (Selvaraj et al., 2005, Nambiar et al., 2012). Protein glycation and oxidation processes share common sites on the protein molecules, which supports a mechanistic relationship (Rondeau and Bourdon, 2011). Oxidative stress and an associated increase in protein glycation is present not only in smokers (Nilsson et al., 1995) but also in chronic renal failure (Sabater et al., 1991), and myocardial infarction (Chowdhury and Lasker, 1998). Our study adds to an increasing body of evidence from various fields, to strengthen our hypothesis that redox status drives protein glycation in non-diabetic individuals.

This study inevitably has limitations. A cross-sectional survey design can only ever be hypothesis-generating, and does not allow for causality to be investigated. We are not claiming that oxidation is the only, or the main mechanism behind protein glycation in non-diabetic and pre-diabetic subjects, but our data do allow us to make the hypothesis that it is playing a part, and a potentially modifiable part. Using proxy measures of oxidative stress and redox status, rather than actual levels of oxidative stress markers (like isoprostanes), is an in-built limitation in this type of general-population health survey. We established the independence of pro- and anti-oxidant environmental factors by including them in the same analytical model. Specific measures could be made in future research. The lack of data on average levels of glycaemia, independent of HbA_{1c} limits the interpretation of the data. Fasting glucose or 2-hour postprandial glucose levels are seldom measured in large epidemiologic studies, and in any case one-off measurements are considered relatively unreliable as reflections of ambient blood glucose. We have explored the possible of effect of small differences in glycaemia by analyses within narrow HbA1c sub-categories, and find the same associations with the indicators of redox status. It would be possible to define the independence of theses associations from those of fasting and 2-hour blood glucose in large diabetes screening datasets which also include measures to reflect redox status, but not in the present study. It is also possible that increased fruit and vegetable intake might be associated with a decreased intake of heat-processed food, a main contributor of dietary AGEs, and an increased consumption of fibre, minerals and other micronutrients. Unfortunately this association could not be investigated in this study due to the nature of the nutritional information available.

3.5 Conclusion

This large population-based study suggests that protein glycation, indicated by HbA1c, is positively associated with smoking, and inversely correlated with vegetable intake. An unfavourable redox status, may thus account for some people having HbA1c in the pre-diabetic range, and this mechanism may promote progression to diabetes, as well as promoting tissue damage. These results strengthen the case for the balance between antioxidant and pro-oxidant status being important in the pathogenesis of chronic diseases.

Chapter 4: Role of oxidative stress in physiological albumin glycation: a neglected interaction

This chapter is presented exactly as published in Free Radical Biology and Medicine

Abstract

Background: Protein glycation is a key mechanism involved in chronic diseases development in both diabetic and non-diabetic individuals. About 12 - 18% of circulating proteins are glycated *in vivo* in normoglycaemic blood, but *in-vitro* studies have hitherto failed to demonstrate glucose-driven glycation below concentration of 30mM.

Methods: Bovine Serum Albumin (BSA), reduced BSA (mercaptalbumin), (both 40g/L) and human plasma were incubated with glucose concentrations 0 - 30mM for 4 weeks at $37^{o}C$. All were tested pre-oxidized for 8 hours prior to glycation with 10nM H_2O_2, or continuously exposed to 10nM H_2O_2 throughout the incubation period. Fructosamine was measured (nitroblue tetrazolium method) at two and four weeks.

Results: Oxidized BSA (both pre-oxidised and continuously exposed to H_2O_2) was more readily glycated than native BSA at all glucose concentrations (p=0.03). Moreover, only oxidized BSA was glycated at physiological glucose concentration (5mM) compared to glucose-free control (glycation increased by 35% compared to native albumin p<0.05). Both 5 and 10mM glucose led to higher glycation when mercaptalbumin was oxidised than un-oxidised (p<0.05). Fructosamine concentration in human plasma was also significantly higher when oxidized and exposed to 5 mM glucose, compared to non-oxidised plasma (p=0.03). The interaction between glucose concentration and oxidation was found to be significant in all protein models (p<0.05).

Conclusion The current study has for the first time demonstrated albumin glycation *in-vitro*, using physiological concentrations of albumin, glucose and hydrogen peroxide, identifying low-grade oxidative stress as a key element early in the glycation process.

4.1 Introduction

Protein glycation is the non-enzymatic glycosylation reaction between reducing sugars and amine residues in proteins (Turk et al., 2001). It is usually considered to be driven by elevated glucose concentrations. Products of this reaction include AGEs which are stable and accumulate in the body where they may exert antigenic effects, and contribute to tissue damage such as atherogenesis (Anderson et al., 1993, Galkina and Ley, 2006). One early-glycation product is glycated hemoglobin (HbA1c), used as a biomarker for the diagnosis of diabetes and monitoring of glucose control in diabetic individuals (Roohk and Zaidi, 2008). Monitoring, and minimizing, glycative damage in diabetic care is of high importance, as glycated proteins and AGEs are implicated in cataract, neuropathy, nephropathy as well as macrovascular diseases (Plutzky, 2011).

However, glycation also occurs in non-diabetic people, in whom up to 6 % of hemoglobin and 12-16% of serum albumin is glycated (Selvin et al., 2011), without exposure to high glucose concentrations (fasting plasma glucose <6mmol/L). Protein glycation heralds tissue damage and function loss, in the normal aging process and as part of the pathogenesis of various chronic diseases. RAGE, found in most tissues, have potent immunomodulatory actions, promoting ROS production and inflammation. Elevated HbA1c can serve as a proxy for both pre-diabetes and metabolic syndrome, as shown in large longitudinal studies (Veeranna et al., 2011). In both non-diabetic and diabetic subjects, HbA1c correlates with coronary heart disease (CHD) risk factors and predicts future CHD and strokes (Myint et al., 2007). A recent study showed that among non-diabetic individuals who did not develop diabetes in the next 3.5 years, those with a higher yet physiological level of HbA1c had higher risk for CVD, in both men and women and after controlling for traditional risk factors (Adams et al., 2009).

The mechanisms leading to protein glycation in the non-diabetic state are not established. The very few *in vitro* studies which have used physiological concentrations are inconclusive as to whether glucose, alone, can successfully promote glycation (Chesne et al., 2006, Bourdon et al., 1999). In diabetic subjects, protein glycation is assumed to be mainly a mass action effect driven by high glucose concentration. However the full process of glycation in diabetes is in fact driven by two separate factors - the concentration of sugars in the initiation phase (mainly glucose, due to its high concentration in blood), and later the pro-oxidant status during Maillard reactions to generate stable AGEs (Fu et al., 1994). We hypothesize that at physiological concentrations of glucose, oxidation may have another role in initiating glycation, supporting earlier speculations from a cross-sectional study in non-diabetic individuals which showed inverse associations between protein glycation and dietary fruit and vegetable consumption, plasma vitamin C and

plasma tocopherol (Bates et al., 2004). Defining an early, preventable oxidative component to the overall glycation mechanism could be of importance in the management of pre-diabetes, when glucose metabolism is only mildly disrupted and glucose-centred clinical approaches might have little effect.

The current study investigates the effect of introducing a mildly pro-oxidative state (hydrogen peroxide at a low physiological concentration of 10nM (Mueller et al., 1997)) on the susceptibility of protein (albumin) to glycation, particularly at physiological and near-physiological glucose concentrations. Another common glycation driver, methylglyoxal, which causes glycative damage in a more oxidative fashion than glucose, was used in physiological concentration both alone and in combination with glucose, to explore possible synergistic effects.

This oxidation-driven glycation hypothesis was also tested on reduced albumin (mercaptalbumin) on the assumption that commercially source native albumin would be already partly oxidized. This work was also extended to proteins in human plasma to extend the physiological relevance of our findings.

4.2 Material and methods

Impact of constant oxidation & pre-oxidation on BSA glycation

Chemicals

Bovine serum albumin (BSA), sodium azide, nitroblue tetrazolium, d-glucose, methylglyoxal, PBS, 1-deoxy-1-morpholinofructose (DMF), hydrogen peroxide, sulphuric acid, dithiothreitol and quinine were purchased from Sigma-Aldrich (Dorset, UK). SnakeSkin Dialysis Tubing, 3.5K MWCO was purchased from Thermo Fisher Scientific (Nottinghamshire, UK).

Glycation of pre-oxidized BSA

BSA (80g/L) was incubated with H_2O_2 (10nM) for 8 hours at 37^oC in PBS and was then dialyzed against PBS (8:1) for another 8 hours. The dialysate was discarded and replaced with fresh PBS three times during dialysis.

To measure the effect of protein pre-oxidation on the susceptibility of BSA to glycation, both native and pre-oxidized albumin (40g/L) were incubated in the presence of glucose (0, 5, 10, 20, and 30mM) for 4 weeks. The combination of methylglyoxal (150nM) and glucose (0, 10 or 20mM) was also studied in order to replicate glycoxidative conditions of albumin in the circulation. All incubations were repeated in 6 replicates.

Albumin glycoxidation

All incubations took place in PBS with sodium azide (0.2g/L) and a final volume of 1.5ml. To investigate the effect of continuous exposure to oxidative damage in the progress of the glycation reaction (glycoxidation) native BSA was incubated with glucose (0, 5, 10, 20 or 30mM) and combinations of methylglyoxal (150nM) plus glucose (0, 10, 20mM) for 4 weeks in the presence of H_2O_2 (10nM). Native BSA was also incubated under the same conditions without H_2O_2 (10nM) to serve as a reference. All incubations were repeated in 6 replicates.

Comparison of the effect of constant glycation among BSA, mercaptalbumin & human plasma

To enhance a concern over the oxidation status of the bovine serum albumin sold by Sigma-Aldrich, the BSA used in the experiments was i) pre-treated with 1.5mM dithiothreitol (DTT) at $37^{o}C$ for 15min and then DTT was removed by extensive dialysis against PBS for 10 hours to create mercapralbumin and ii) BSA as bought from Sigma-Aldrich.

In order to investigate the effect of hydrogen peroxide exposure in plasma proteins glycation, pooled plasma from 8 healthy, normal weight volunteers was collected in heparin tubes after an overnight fast.

Protein glycoxidation

All incubations took place in PBS with sodium azide (0.2g/L) and a final volume of 1.5ml. To investigate the effect of constant exposure to oxidative damage in the progress of the glycation reaction (glycoxidation) native BSA and native mercaptalbumin was incubated with glucose (0, 5, 10 or 20mM) for 4 weeks in the presence of H_2O_2 (10nM). Proteins were also incubated under the same conditions without H_2O_2 (10nM) to serve as a reference.

The glucose concentration of 30mM was not employed as it is a highly supra-physiological concentration and the combinations of methylglyoxal plus glucose were also not employed.

All incubations were repeated in 5 replicates.

Fructosamine measurement

The NBT assay was modified in this experiment using a larger amount of sample (25 vs 10μL) which resulted in a smaller Coefficient of Variation.

Fructosamine levels were measured at week 2 and 4 with the NBT assay, performed in microplates as described previously (Baker et al., 1993). Briefly, samples (25μL) were added to of sodium carbonate buffer (100μL, 100mM, and pH 10.8) with Nitroblue Tetrazolium (0.25mM). Microplates were incubated for 15min at $37^{o}C$ and measured spectrophometrically against

controls at 550nm after 10 and 15min of incubation. The difference between the two readings was used to calculate concentrations. The fructosamine analog 1-deoxy-1-morpholinofructose (DMF) was used as a standard. All fructosamine measurements were performed in duplicate. Standards and NBT reagent were made fresh every week and stored at -20°C and 4°C respectively. All samples were stored at -20°C.

Statistical analysis

All combinations of oxidative damage and glycation drivers were tested as five or six true replicates, according to the experiment. The independent sample t-test was used to assess the differences in glycation between native BSA and either of the oxidation set-ups. Difference in fructosamine production between glucose levels and glycation drivers were tested using a one-way ANOVA and Tukey's post-hoc test. Differences between exposure to oxidation and no oxidation were studied in each protein system separately using one-way ANOVA and the interaction between glucose levels and oxidation was studied using two-way ANOVA. Statistical analysis was performed using PASW 18.

4.3 Results

Effect of constant oxidation on BSA glycation

Incubation of native BSA (40g/L) with glucose concentrations below 20mM did not lead to measurable levels of glycated BSA measured after 2 weeks, with or without 150nM methylglyoxal, compared to glucose-free control (Table 4.1). After 4 weeks, incubation with 10mM glucose (with or without methylglyoxal) significantly promoted glycation in native BSA compared to glucose-free control. Exposure to a physiological concentration of hydrogen peroxide (10nM), however, led to significantly higher glycated BSA (measured as fructosamine) at the lower glucose concentrations of 10mM after 2 weeks, and 5 mM after 4 weeks incubation (both p<0.05 vs. glucose free control).

Table 4.1 Fructosamine concentration after two weeks incubation of albumin with different glucose concentrations, between and within treatments (glucose / MGO exposure and oxidation)

Glucose levels (mM)	Oxidation status					
	Week 2					
	Native Mean (SD)	H_2O_2 10nM Mean(SD)	p-value	Native Mean (SD)	Pre-Oxidized Mean(SD)	p-value
0	0.26(0.04)	0.27(0.02)	0.669	0.26(0.01)	0.26(0.03)	0.807
5	0.31(0.04)	0.38(0.02)	0.017	0.23(0.02)	0.29(0.05)	0.016
10	0.35(0.02)	0.53(0.02)*	0.001	0.26(0.04)	0.28(0.03)	0.177
20	0.51(0.05)*	0.69(0.07)*	0.002	0.57(0.06)*	0.57(0.04)*	0.940
30	0.82(0.06)*	0.82(0.03)*	0.987	0.79(0.10)*	0.64(0.10)*	0.041
0+MGO	0.26(0.02)	0.31(0.01)	0.015	0.19(0.02)	0.19(0.03)	0.510
10+MGO	0.34(0.05)	0.46(0.03)*	0.001	0.62(0.07)*	0.48(0.05)*	0.002
20+MGO	0.53(0.08)*	0.67(0.01)*	0.008	0.46(0.05)*	0.39(0.07)*	0.048
Oxidised vs native[#]			<0.001			0.005
	Week 4					
0	0.37(0.03)	0.39(0.09)	0.767	0.20(0.02)	0.17(0.02)	0.021
5	0.40(0.09)	0.54(0.06)*	0.015	0.26(0.02)	0.24(0.02)*	0.063
10	0.55(0.04)*	0.68(0.06)*	0.003	0.31(0.02)*	0.31(0.02)*	0.825
20	0.78(0.04)*	0.92(0.04)*	<0.001	0.56(0.05)*	0.52(0.07)*	0.292
30	0.95(0.08)*	1.17(0.10)*	0.002	0.62(0.05)*	0.64(0.03)*	0.844
0+MGO	0.43(0.02)	0.40(0.04)	0.590	0.22(0.02)	0.17(0.01)	0.016
10+MGO	0.70(0.03)*	0.84(0.09)*	0.010	0.52(0.01)*	0.46(0.03)*	0.028
20+MGO	0.86(0.07)*	1.05(0.12)*	0.015	0.40(0.02)*	0.33(0.01)*	<0.001
Oxidised vs native[#]			<0.001			<0.001

*$p<0.05$ vs. glucose 0mM, [#] two-way ANOVA analysis

Using constantly-oxidized BSA generated significantly more fructosamine than native BSA after 2 weeks of incubation with 5, 10, and 20mM glucose (increased by 23%, 36% and 35% respectively). Similar results were observed with methylglyoxal (150nM) alone (19% increase in fructosamine), and with a combination of methylglyoxal (150nM) plus glucose at concentrations of 10 and 20mM, with 35% and 26% increases in fructosamine respectively (Figure 4.1). Significantly greater glycation of constantly-oxidized BSA compared to native BSA was also observed after 4 weeks at all glucose concentrations, and with combinations of glucose (10 & 20mM) and methylglyoxal (150nM) (Figure 4.2). In particular, incubation of BSA with 5mM glucose and 10mM H_2O_2 led to a 35% higher fructosamine concentration compared to the non-oxidised control (p=0.04). Although incubating BSA in presence of methylglyoxal (150nM) alone did not lead to significantly increased glycation after neither 2 nor 4 weeks (Table 4.1), combining methylglyoxal (150nM) and glucose (10mM) had a synergistic effect on glycation of constantly-oxidised BSA after 4 weeks (p=0.02 vs. glucose alone), as well as some suggestion of an effect on native BSA (p=0.08) (data not shown).

The individual impacts of glucose concentration and oxidation, as well as their interaction, on glycation over periods of 2 and 4 weeks were investigated using a two-way ANOVA. Oxidation had a significant effect on glycation at both two and four weeks (p<0.001) There was strong evidence for an interaction between continuous-oxidation and glucose concentration in driving glycation after 2 weeks of incubation (p<0.001) with a non-significant indication of an effect of this interaction on glycation after 4 weeks (p=0.058). While removing data relating to methylglyoxal and glucose incubations from the analysis did not change impact on the significance of the effect of oxidation on protein glycation (p <0.001 at both two and four weeks), the interaction between oxidation and glucose concentration significantly affected glycation after both two and four weeks (p=0.001 and p=0.01, respectively).

Figure 4.3 Fructosamine concentration (mM DMF equivalent) after two weeks incubation with glucose and constant exposure to oxidation from hydrogen peroxide (10nM).
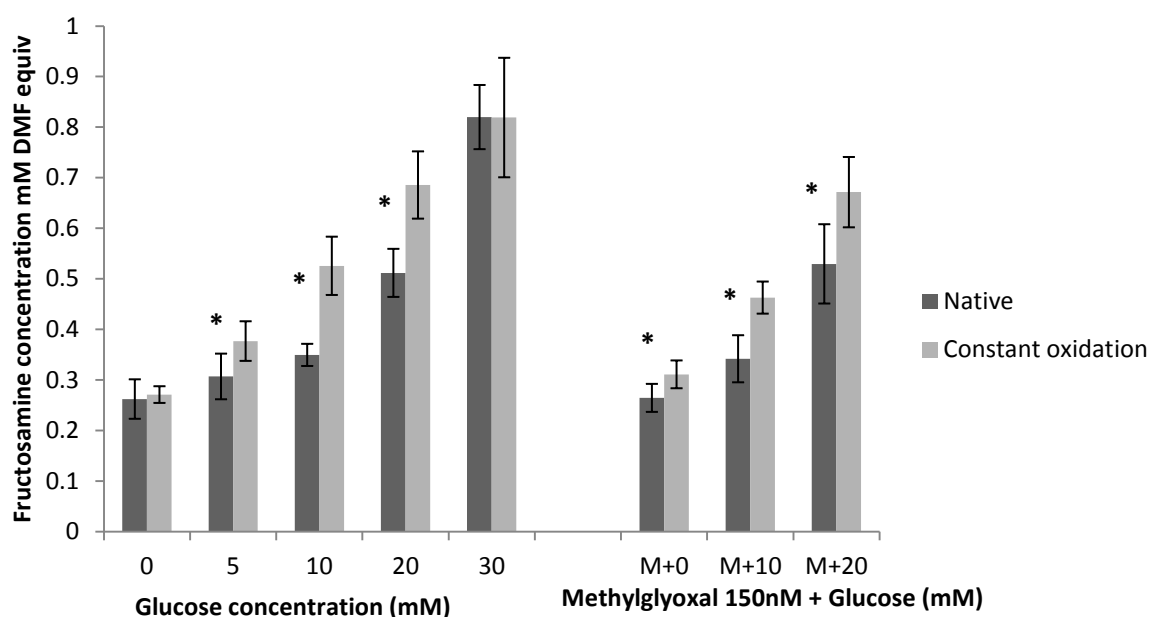
Two-way ANOVA analysis showed a significant effect of oxidation to promote glycation.*p<0.05 native vs. constant oxidation for each given glucose concentration

Figure 4.4 Fructosamine concentration (mM DMF equivalent) after four weeks incubation with glucose and constant exposure to oxidation from hydrogen peroxide (10nM).
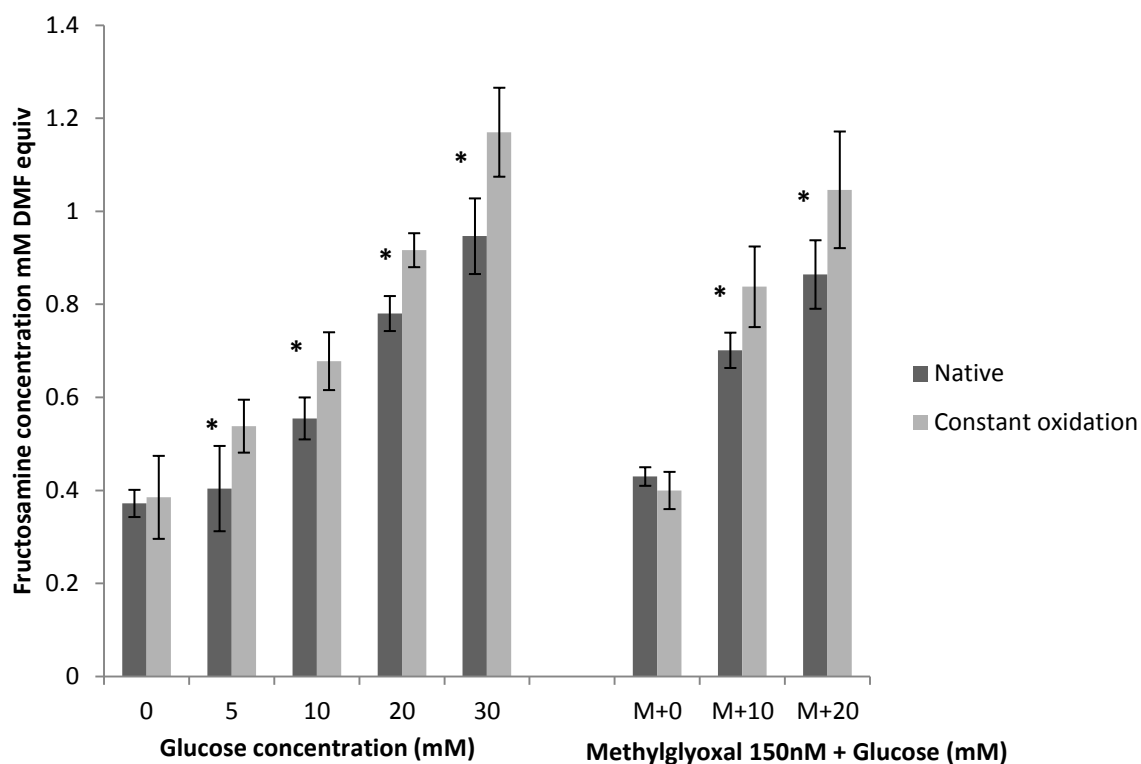
Two-way ANOVA analysis showed a significant effect of oxidation to promote glycation.*p<0.05 native vs. constant oxidation for each given glucose concentration

Effect of pre-oxidation on BSA glycation

Incubation of native and pre-oxidised BSA with glucose concentrations lower than 20mM for two weeks did not lead to significantly more fructosamine being produced than the glucose-free control. Nonetheless the pre-oxidation step led to significantly higher glycation, compared to native BSA, after two weeks at the lowest glucose concentration (5mM) (p=0.016).

A 4-week incubation with 5mM glucose alone was sufficient to drive glycation of pre-oxidized BSA (p=0.03 vs. glucose-free control), but not native (un-oxidized) BSA which required at least 10mM glucose (p=0.001, Table 4.1).

Comparison of the effect of constant-oxidation on BSA and mercaptalbumin glycation

Reduction of BSA to mercaptalbumin was employed in order to investigate whether commercially available BSA, possibly being oxidised to some extent, would be more or less prone to subtle oxidation driven glycation. For this reason, DTT-treated BSA was incubated with glucose (0-20mM) in presence or absence of 10nM H_2O_2.

When mercaptalbumin was incubated with 5 and 10mM glucose under constant oxidation for two weeks, significantly higher fructosamine levels were observed compared to non-oxidised mercaptalbumin (p=0.03 & p=0.006; respectively). While incubation for two weeks with 5mM glucose was sufficient to drive higher glycation in constantly-oxidized mercaptalbumin than glucose-free control (p<0.001), non-oxidised mercaptalbumin required incubation with 10mM glucose to lead to higher glycation than the glucose-free control (p<0.001). No differences between oxidized and non-oxidised mercaptalbumin were observed at week 4 (Figure 4.3).

Two-way ANOVA, analysing the effect of oxidation at all glucose levels, showed that oxidized mercaptalbumin was subject to higher glycation than non-oxidised, at both weeks two and four (p=0.003 & p=0.035 respectively). The interaction between glucose and oxidation was not significantly affecting glycation in both weeks (p for interaction glucose × oxidation = 0.48 & 0.78 for week 2 and 4 respectively).

Figure 4.5 Fructosamine concentration (mM DMF equivalent) after two and four weeks incubation with glucose and constant exposure to oxidation from hydrogen peroxide (10nM) in mercaptalbumin.

Mercaptalbumin

Week 2
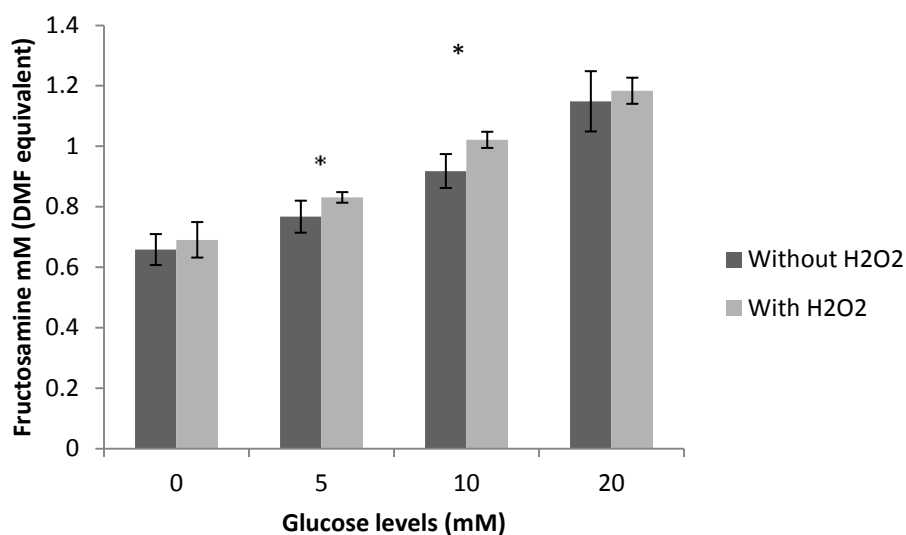
Week 4

Two-way ANOVA analysis showed a significant effect of oxidation to promote glycation.*$p < 0.05$ native vs. constant oxidation

When mercaptalbumin glycation was compared to BSA glycation. Mercaptalbumin was more prone to glycation than BSA in both the presence and absence of H_2O_2. In the absence of H_2O_2 mercaptalbumin had higher fructosamine concentration than BSA at 5 and 10mM glucose at two weeks (p=0.004 & p=0.002 respectively) and that was significant at week 4 for 5mM glucose (p=0.005) and nearly significant for 10mM glucose (p=0.06). In the presence of H_2O_2 mercaptalbumin was again more successfully glycated than BSA at 5&10 mM glucose at week 2 (p<0.001, for both) and nearly significantly more at 20mM (p=0.057). At week 4 mercaptalbumin was significantly more glycated than BSA only at 5mM glucose (p=0.04) (data not shown).

Employing two-way ANOVA showed that mercaptalbumin was more prone to glycation than BSA (p<0.001) and there was a significant positive interaction between oxidation and the type of protein employed, in favour of mercaptalbumin (p interaction protein-type x oxidation= 0.047) at week 2. Although the interaction between the protein type and oxidation was not documented at week 4 (p=0.33), glycation was still positively affected by using mercaptalbumin rather than BSA (p<0.001) (data not shown).

Effect of constant-oxidation on human plasma glycation

Protein glycation in human plasma was studied to explore the reactions studied previously in a more complex protein system with antioxidant mechanisms in place and closer to human physiology. Plasma exposure to constant hydrogen peroxide (10nM) promoted glycation when incubated with 5mM glucose for 2 weeks, compared to non-oxidised plasma (p=0.03). Surprisingly, this effect of oxidation was no present after 4 weeks, and actually led to significant lower fructosamine concentration for incubations with 10mM glucose compared to non-oxidised plasma (p=0.001) (Figure 4.4).

Two-way ANOVA suggested that oxidation had no impact on glycation in human plasma at week two but it had a significant negative impact at week four (p=0.01). The interaction glucose × oxidation was also significant (p<0.001) at week four, only.

Figure 4.6 Fructosamine concentration (mM DMF equivalent) after two and four weeks incubation with glucose and constant exposure to oxidation from hydrogen peroxide (10nM) in human plasma.

Human Plasma

Week 2
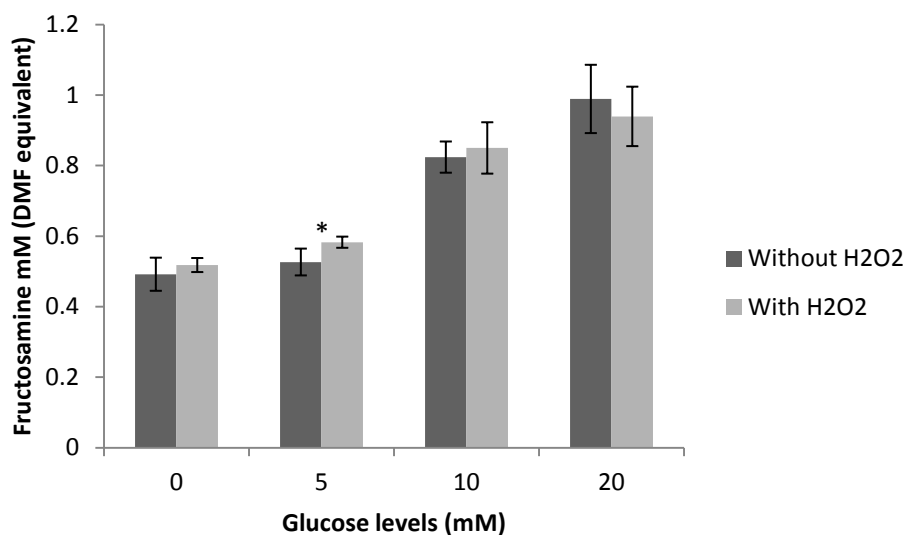
Week 4
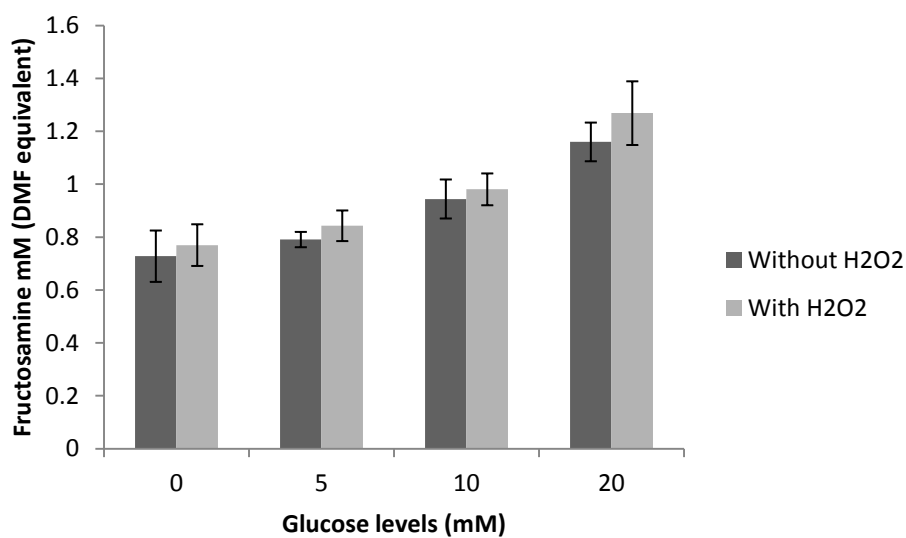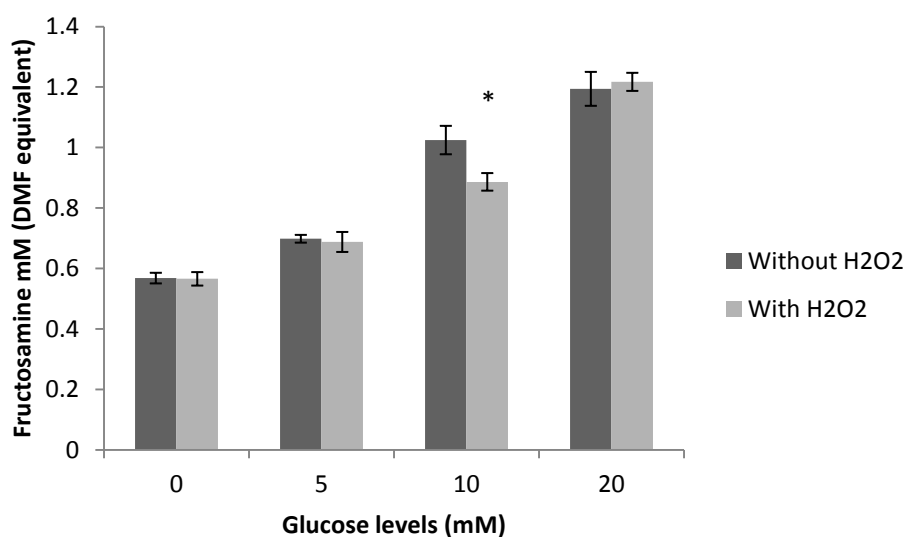

Two-way ANOVA analysis showed a significant effect of oxidation to promote glycation.*$p < 0.05$ native vs. constant oxidation

4.4 Discussion

Protein glycation was first described as part of the Maillard reactions cascade, as a cause of food-spoiling. Although the same reactions have been identified *in-vivo,* and glycated proteins are probably causally associated with the tissue damage that occurs during aging, in diabetes and other chronic diseases (Plutzky, 2011), there are still gaps in the understanding of the exact mechanisms involved. It is striking that 12-18% of circulating albumin, and up to 6% of haemoglobin is glycated in apparently healthy non-diabetic people, amongst whom most heart disease occurs (Selvin et al., 2011).

Perhaps surprisingly, this study is the first to demonstrate *in vitro* protein glycation at physiological glucose concentrations. The clear effect of including an oxidative agent (hydrogen peroxide at a low, physiologically relevant concentration (Mueller et al., 1997)), supports the concept of oxidative stress as a key mechanism behind *in-vivo* glycation of albumin in normoglycaemic individuals. It is worth stressing that the concentration of hydrogen peroxide used was very low. Although the literature is still controversial over the exact concentration of hydrogen peroxide in plasma with values up to 35µM being documented (Varma and Devamanoharan, 1991), even the supporters of the theory that hydrogen peroxide concentration is not important in plasma documented values of 250nM (Frei et al., 1988), which is 25 times higher than the concentration equipped in the current study. Several factors might explain why previous studies have failed to achieve glycation *in vitro* under physiological conditions. We used a physiological concentration of albumin, while previous studies have used lower (sub-physiological) concentrations (0.01 -7g/L) (Gugliucci et al., 2009, Tarwadi and Agte, 2011), and/or high (supra-physiological) glucose concentrations (30mM - 0.5M) (Harris et al., 2011, Verzelloni et al., 2011a). Albumin glycation had previously been achieved in the presence of 15mM glucose alone at 5 weeks or 30mM glucose alone at 4 weeks (Barnaby et al., 2011). With physiological glucose concentrations, results have varied according to sample treatment and methods used to measure glycation. Bourdon et al. (1999) reported that incubation of albumin for 4 weeks with 5mM glucose did not promote glycation, while later reporting contradictory findings (with, however, glycation only implied from qualitative results (Chesne et al., 2006)).

Similarly, while methylglyoxal is a potent glycative molecule in supra-physiological conditions (Tarwadi and Agte, 2011b), it did not show significant glycative activity in physiological concentrations. Our data suggest it might act synergistically with glucose to promote glycation at lower glucose concentrations (10mM), but we demonstrated that both glucose and methylglyoxal at physiological concentrations will glycate albumin if it is oxidised.

In both the BSA and mercaptalbumin models, glycation of the protein was significantly higher than the glucose-free control when exposed to a physiological concentration of hydrogen peroxide for two weeks. Although the effect of pre-oxidation and continuous oxidation were very similar with a favour towards continuous oxidation, in so low hydrogen peroxide concentrations the continuous oxidation model is more likely to be of physiological relevance. Continuous exposure to hydrogen peroxide led to higher fructosamine concentrations at all glucose levels and oxidation was also shown to act synergistically with glucose, as the interaction between the two was found to be significant in our experiments.

Hydrogen peroxide positively interacts with glucose in promoting glycation reactions. One has to keep in mind that the hydrogen peroxide exposure was weak in term of concentration in order to resemble physiological condition and hence it is likely to induce important but subtle effects. When human plasma was exposed to hydrogen peroxide, glycation was significantly higher with 5mM glucose after two weeks, compared to the non-oxidized control. The opposite was found when oxidised plasma was incubated with 10mM glucose for 4 weeks, but the lower fructosamine concentration of the oxidised plasma in that case could be attributed both to increased protein instability and/or glycation being driven to the production of AGEs (not detectable by the NBT method used) rather than early-glycation products as fructosamine.

Constant oxidative stress is clearly damaging and relevant to diabetic and obese chronic pro-oxidant states, however a dynamic balance between pro- and anti-oxidant factors is usually present in plasma and other body fluids. The level of oxidative stress fluctuates during the day (e.g. higher post-prandially) and a variety of events can trigger short-term production of Reactive Oxygen Species. Our results suggest that episodes of relatively unopposed oxidation, e.g. from infection or inflammation, or smoking, could damage proteins to promote subsequent glycation, as we have demonstrated with the increased susceptibility of pre-oxidized albumin to glycation at a physiological glucose concentration. This mechanism could apply *in vivo*.

Recognizing that, in physiological systems, protein glycation depends on oxidative damage as well as glucose concentration has implications for scientific understanding and potentially for clinical practice. The term 'glycoxidation', currently restricted to the latter stages of Maillard reactions, seems more appropriate than simply 'glycation' to describe the overall *in vivo* protein glycation process, and similar protocols to ours would be appropriate to study the phenomenon *in vitro*. The quest for normoglycaemia in diabetes management is important to delay vascular and other complications, but potentially hazardous interventions are entirely directed at glucose-lowering: using insulin or anti-diabetic drugs intensively to reduce glycation has been associated with increased risk of hypoglycemia, and of mortality (Terry et al., 2012). If oxidative stress is also involved as a trigger for protein glycation and tissue damage, then approaches aimed solely at

glucose handling are insufficient, and reducing oxidative stress might be less hazardous. This is not arguing for indiscriminate or high-dose antioxidant treatments. Several trials have suggested worse outcomes from antioxidant vitamin supplementation (Virtamo et al., 2003), leading to understandable prejudice against their effectiveness and safety, but a recent meta-analysis of 66 randomised controlled trials indicates benefit from vitamin E supplementation for primary prevention (where there is some baseline insufficiency) (Biesalski et al., 2010), and vitamin E also contributes to secondary prevention (Stephens et al., 1996), renewing interest in antioxidant interventions. Our results support findings from the cross-sectional study of Bates et al. (2004), which led to the hypothesis that dietary antioxidants may reduce tissue glycation. Also evidence from *in-vitro* studies suggests that antioxidants are having a protective role in protein glycation (Harris et al., 2011, Vinson and Howard, 1996). Exposure to oxygen radicals such as TBH and H_2O_2 significantly increased haemoglobin glycation *in-vitro* and pre-treatment with vitamin E blocked that effect (Jain and Palmer, 1997). Replenishment of the antioxidant defences of GSH-deficient red blood cells, on the other hand, protected them against increased haemoglobin glycation (Jain, 1998), both supporting the hypothesis of oxidative stress being involved in protein glycation.

The present studies suggest some important avenues for future research, as well as changes to commonly-used experimental models. It is important to question accepted patho-physiological mechanisms if they cannot be demonstrated *in vivo* at physiological concentrations. Our evidence that mild oxidation plays an early role in AGE production is novel and explains a gap in the literature. Prior reduction of BSA might indeed increase the oxidation potential of the protein and hence strengthen the effect of oxidation on glycation, as shown by the two-way ANOVA analysis. While being designed to replicate physiological conditions and employing a large number of replicates (6 instead of the usual 3) to reduce random errors under physiological conditions, the current study does have limitations. Hydrogen peroxide's stability in the incubation media was not tested, which allows for the possibility of the molecule having a shorter half-life than the experimental duration. Nonetheless the effects of exposure to hydrogen peroxide, no matter how long, are carried over the 4-week experimental period. In circulation hydrogen peroxide would be continuously produced and removed leading to fluctuations and a continuous exposure of the protein to oxidative damage, likely to amplify the effects seen in this experimental study. Albumin, although the major circulating protein, may not be representative of other glycation-prone proteins, and the results cannot provide an exact mechanism linking oxidative damage to glycation. Using human plasma led to slightly different results than BSA and mercaptalbumin. No effect on glycation was seen from oxidation at glucose levels above 10mM after two weeks of incubation; that could be attributed to the fact that plasma from healthy volunteers involves different proteins with different degrees of pre-oxidation and glycation and also a much more competent antioxidant system which would be expected to rapidly scavenge ROS. Glycated

proteins already present in plasma could also affect the speed and general kinetics of the reaction. Finally, multiple testing could have resulted in chance findings of 'significance' but since for every condition there were no more than 7 tests ran, p-values lower than 0.007 should be considered as true findings. Possible mechanisms involve protein damage by hydrogen peroxide and/or increased glucose autoxidation in the presence of hydrogen peroxide, both likely to increase the affinity of the molecules for the non-enzymatic sugar linkage. There may be selective oxidation of amino acids: i.e., tryptophan, a main site for protein glycation, is an oxidation site for human albumin (Rondeau and Bourdon, 2011, Guerin-Dubourg et al., 2012), suggesting that oxidized amino acids maybe more susceptible to further glycative damage.

4.5 Conclusion

Oxidative damage, although known to be important for the late stages of protein glycation, has not previously been linked with the early stage of the Maillard reaction. Our data suggest that oxidative damage, induced by a very low (physiological) concentration of hydrogen peroxide, plays a critical early role in fructosamine production. Importantly, the effect is seen at physiological glucose concentrations, potentially opening an avenue for new preventive treatments. Our experiments highlight the importance of oxidative stress on protein glycation, as a promoter and even a necessary condition to achieve glycation in physiological glucose concentrations.

Chapter 5: Inhibition of protein glycation by phenolic acids: physiological relevance and implication of protein-phenolic interactions

This chapter is presented exactly as published in Food & Function

Abstract

Background: While antiglycative capacity has been attributed to (poly)phenols, the exact mechanism of action remains unclear. Studies so far are often relying on supra-physiological concentrations and use of non-bioavailable compounds.

Methods: To inform the design of a physiologically relevant *in-vitro* study, we carried out a systematic literature review of dietary interventions reporting plasma concentrations polyphenol metabolites. Bovine Serum Albumin (BSA) was pre-treated prior to in vitro glycation: either no treatment (native), pre-oxidised (incubated with 10nM H_2O_2, for 8 hours) or incubated with a mixture of phenolic acids at physiologically relevant concentrations, for 8 hours). *In-vitro* glycation was carried out in presence of i) glucose only (0, 5 or 10mM), ii) glucose (0, 5 or 10mM) plus H_2O_2 (10nM), or iii) glucose (0, 5 or 10mM) plus phenolic acids (10-160nM). Fructosamine was measured using the nitroblue tetrazolium method.

Results: Following (high) dietary polyphenol intake, 3-hydroxyphenylacetic acid is the most abundant phenolic acid in peripheral blood (up to 338μM) with concentrations for other phenolic acids ranging from 13nM-200μM. Presence of six phenolic acids with BSA during *in-vitro* glycation did not lower fructosamine formation. However, when BSA was pre-incubated with phenolic acids, significantly lower concentration of fructosamine was detected under glycoxidative conditions (glucose 5 or 10mM plus H_2O_2 10nM) (p<0.001 vs. native BSA).

Conclusion: Protein pre-treatment, either with oxidants or phenolic acids, is an important regulator of subsequent glycation in a physiologically relevant system. High quality *in-vitro* studies under conditions closer to physiology are feasible and should be employed more frequently.

5.1 Introduction

Protein glycation has been implicated in the development of several chronic diseases, particularly diabetic micro and macrovascular complications (Sabanayagam et al., 2009, Yan et al., 2003). The process of glycation, in diabetes, is mainly driven by the elevated blood glucose concentration through non-enzymatic condensation of a sugar molecule on a protein, lipid or DNA molecule (Baynes, 2002, Thornalley, 2003a). Measurement of glycated haemoglobin is the standard method for monitoring diabetes control (American Diabetes, 2011) and elevated levels are clearly related to tissue damage. Glycated albumin levels in plasma can vary widely, between 1% to up to 16% in normoglyceamic individuals (Shaklai et al., 1984, Selvin et al., 2011) and the reasons for this wide range are not fully understood. In individuals free of diabetes for every 1% increase in HbA1c levels there is an associated 30-50% in the risk of cardiovascular disease (Selvin et al., 2010, Barr et al., 2009, Selvin et al., 2005). Although *in-vitro* studies have been employed to study glycation mechanisms, few have used physiologically relevant glucose concentrations. *In vitro* protein glycation does not easily occur with physiological concentrations of glucose, implying that another factor was necessary. We recently demonstrated that albumin glycation at physiological glucose concentrations (5 and 10mM) was driven by oxidative stress, and that oxidised albumin is more susceptible to glycation than the native form of the protein (Vlassopoulos et al., 2013b). We have suggested that the reaction might be considered protein glycoxidation, rather than simply glycation. The study of early glycation, fructosamine, in *in-vitro* model is of high importance both for translational value (HbA1c is commonly used in clinical practice) and due to the fact that it is the first stable glycation product and so its production rate is influential on AGEs production(Venkatraman et al. 2001).

Antioxidants, and (poly)phenols and their metabolites in particular, have been studied for their *in-vitro* antiglycative properties (Xie and Chen, 2013, Kim and Kim, 2003, Hou et al., 2013, Wu et al., 2009, Sompong et al., 2013). (Poly)phenols may offer protection by scavenging ROS produced during the glycation reaction, thereby slowing glycation and inhibiting the formation of AGEs (Wu and Yen, 2005, Xie and Chen, 2013). Another possible mechanism involves "physical" protection against glycation. This mechanism suggests that (poly)phenols have the capacity to bind on the protein molecule, most likely with a non-covalent bond, and in this way make glycation targets on the protein molecule (usually amino acids like lysine) inaccessible to take part in the glycation reaction (Xiao and Kai, 2012, Verzelloni et al., 2011b).

While these studies may hold value in food science (for example, reduction of AGEs formation during cooking in the presence of polyphenols (Zhang et al., 2014)), their physiological relevance to human health is sometimes questionable. (Poly)phenols are subject to extensive metabolism in

the lumen (hydrolysis by the enterocytes' glucosidase system) and after absorption (glucuronidation/sulfation in the liver) (Scalbert et al., 2002, Manach et al., 2005). Most high molecular weight dietary polyphenols have low bioavailability and even though aglycones may reach systemic circulation in small amounts, glycosides do not (Nagasawa et al., 2003, Williamson and Manach, 2005, Manach et al., 2005, Crozier et al., 2009). Those which do not get absorbed instead accumulate in the colon lumen, where they are subject to bacterial degradation, leading to the formation of the phenolic acids. Phenolic acids have a relatively higher bioavailability (Scalbert et al., 2002, Scalbert and Williamson, 2000). In plasma, an increase in phenolic acid levels is seen 8-10 hours after ingestion, which represents the 'colonic tide' of (poly)phenol metabolites (Manach et al., 2004, Scalbert et al., 2002, Vitaglione et al., 2007). Studies using foodstuff extracts and mixtures containing aglycones and glycosides thus do not replicate physiology (Williamson and Manach, 2005, Harris et al., 2014) when (poly)phenols in the circulation are mostly phase II metabolites and rarely exceed $1\mu M$ concentration (and if so, transiently) (Manach et al., 2004, Scalbert and Williamson, 2000). Finally, while single compound studies are informative and allow for mechanisms of action to be dissected, polyphenols and their metabolites are not found or consumed in isolation (Crozier et al., 2009, Manach et al., 2005); they are, also, all consumed within complex food matrices with other nutrients that may modify absorption and metabolism (Serafini et al., 2009, Mullen et al., 2008).

The present paper systematically reviews the literature reporting plasma levels of phenolic acids as key polyphenol metabolites, following ingestion of polyphenol rich food products (not under "acute" trial settings). The outcomes of this review are then used to test whether phenolic acids can inhibit the early stages of glycation under physiologically-relevant experimental conditions, using the bench-top design we previously described (Vlassopoulos et al., 2013b).

5.2 Material and methods

Systematic literature review

This review was conducted following the Preferred Reporting Items for Systematic Review and Meta-Analysis (PRISMA) guidelines. A literature research was carried out in PubMed® and ISI Web of Knowledge® for trials reporting plasma phenolic acid levels after a high polyphenol food/diet intake. The search was inclusive of all years up to February 2014. The following search terms were used to identify relevant studies: (phenol*, polyphenol*, phenolic acid*, fruit, vegetable, spice, cocoa, herb, juice, oil, wine, extract, tea or coffee), paired (bolean AND) with (feeding, trial, intervention, consumption OR supplementation). The wild-card term "*" was used to improve the sensitivity of the search by increasing the number of matches. The review was limited to studies utilising chromatographic techniques to identify (poly)phenolic compounds in serum or plasma. Studies on animals were excluded, as well as studies reporting cross-sectional data. Only controlled long-term feeding trials were reviewed. Studies were included in the review if absolute concentrations of phenolic acids were reported.

Experimental procedures

Chemicals

Bovine serum albumin (BSA), sodium azide, nitroblue tetrazolium, d-glucose, PBS, 1-deoxy-1-morpholinofructose (DMF), hydrogen peroxide, caffeic acid, p-coumaric acid, vanillic acid, 3-hydroxyphenylacetic acid, 3,4-dihydroxyphenylacetic acid and protocatechuic acid were purchased from Sigma-Aldrich (Dorset, UK). SnakeSkin Dialysis Tubing, 3.5K MWCO was purchased from Thermo Fisher Scientific (Nottinghamshire, UK).

Albumin pre-treatment

BSA (40g/L) was studied in three different forms: native BSA (BSA), pre-oxidised BSA (ox-BSA), and phenolic acid-preincubated BSA (PP-BSA). Ox-BSA was incubated with 10nM hydrogen peroxide (H_2O_2) for 8hrs pre-glucose incubation and PP-BSA was incubated with a phenolic acid mixture for 8hrs pre-glucose incubation. The phenolic acids used were selected based on the results of the literature review aiming to be representative of plasma concentrations of free phenolic acids, with a higher degree of physiological relevance. The acids and concentrations used were: caffeic acid 10nM, p-coumaric acid 8nM, vanillic acid 21nM, protocatechuic acid 40nM, 3-hydroxyphenyl acetic acid 160nM and 3,4-dihydroxyphenyl acetic acid 40nM. Following pre-treatment, ox-BSA and PP-BSA were dialysed against PBS for 24 hrs to remove any free H_2O_2 and/or phenolic acids (Figure 5.1).

Protein glycation

All incubations took place in PBS (0.137M Sodium chloride, 0.0027M potassium chloride, and 0.010M phosphates) with sodium azide (0.2g/L), in a final volume of 1.5ml, for 14 days. BSA, ox-BSA and PP-BSA (40g/L) were incubated with glucose (0, 5, 10mM) with or without 10nM H_2O_2 or a physiologically relevant phenolic acid mix (as described above). All incubations were replicated 6 times.

Figure 5.7 Experimental design for the study of the antiglycative potential of physiologically relevant phenolic acids.
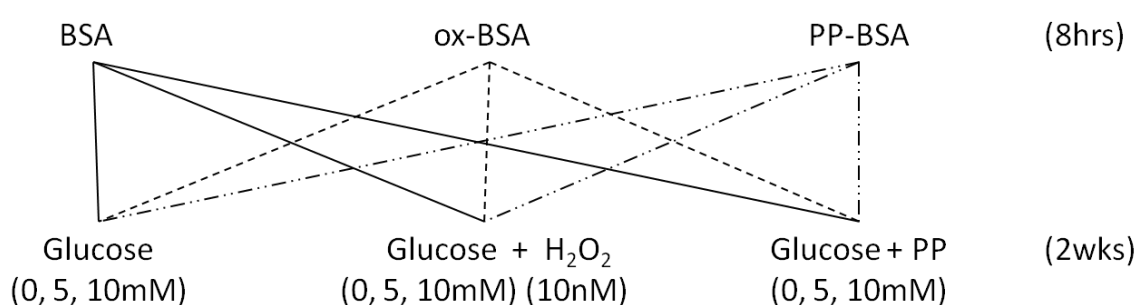
BSA: bovine serum albumin, ox-BSA: bovine serum albumin incubated with 10 nM H_2O_2, PP-BSA: bovine serum albumin incubated with phenolic acids mixture; PP: caffeic acid 10nM, p-coumaric acid 8nM, vanillic acid 21nM, protocatechuic acid 40nM, 3-hydroxyphenyl acetic acid 160nM and 3,4-dihydroxyphenyl acetic acid 40nM

Fructosamine measurement

Fructosamine levels were measured at week 2 with the NBT assay, performed in microplates as described previously [34]. Briefly, samples (30μL) were added to of sodium carbonate buffer (100μL, 100mM, and pH 10.8) with Nitroblue Tetrazolium (0.25mM). Microplates were incubated for 15 min at 37°C and measured spectrophometrically against controls at 550nm after 10 and 15 min of incubation. The difference between the two readings was used to calculate concentrations. The fructosamine analog 1-deoxy-1-morpholinofructose (DMF) was used as a standard. All fructosamine measurements were performed in duplicate. Standards and NBT reagent were made fresh every week and stored at -20°C and 4°C respectively. All samples were stored at -20°C prior to assay.

Measurement of AGE fluorescence

AGE fluorescence was measured in diluted samples (1:5) at an excitation wavelength 370nm and an emission wavelength 440nm. AGE fluorescence was measured as arbitrary units and in duplicate using a SpectraMax M2 plate reader.

Protein structure analysis

Protein tryptophan fluorescence intensity was recorded with a Shimadzu RF-5301 PC Spectrofluorophotometer, using 5nm excitation and 10nm emission slit widths. Proteins at a concentration of 0.2g/L in PBS were used after dialysis and prior to glycation. The UV spectra of the protein, measured using a Perkin Elmer Lambda 25 UV/VIS Spectrometer, indicated a maximum absorbance at 277nm for all proteins, which was thereafter used as the excitation wavelength for each protein. Changes in maximum emission were compared between proteins in a semi-quantitative manner and changes in the emission spectra due to pre-treatment were also compared qualitatively. Near UV Circular Dichroism spectra of 1.5g/L protein and far UV Circular Dichroism spectra of 0.2g/L protein were recorded in a 0.5cm and 0.02 cm pathlength quartz cuvette, respectively, using a Jasco J-810 spectropolarimeter [35]. The UniProt database was used to identify structures, sequences and drug binding location on the BSA molecule (http://www.uniprot.org/uniprot/P02769).

Statistical analysis

All combinations of oxidative damage and glycation drivers were tested as six true replicates, according to the experiment. Assays were conducted in duplicate. Differences in fructosamine production between hydrogen peroxide levels were tested using a one-way ANOVA and Tukey's post-hoc tests, at each glucose level separately. The interaction between glucose levels and hydrogen peroxide levels, as well as the overall dose response effect, were studied using two-way ANOVA. Statistical analysis was performed using SPSS statistical software package version 19.0.0 (IBM, SPSS Software, Armonk, NY, USA).

5.3 Results

Using the primary search terms, 436 papers were identified, with 40 excluded as duplicate entries. Titles from the remaining 396 articles were screened and 43 were excluded as not relevant to the topic of the review (mostly studies focusing on phenylalanine and other phenol ring-containing substances). During abstracts screening, 246 reports were removed from the analysis with the majority being in-vitro or animal studies' reports, alongside with reports from cross-sectional studies. Review papers, conference proceedings, and reports written in languages other than English were also excluded. A total of 107 full papers were screened, leading to the exclusion of 28 additional reports. These reports either performed a qualitative analysis of

metabolites in plasma or did not provide data on the concentrations of the metabolites measured in plasma. The reference list of each publication was screened in order to identify other publications from the same study, in case the data on the metabolites concentrations were published elsewhere. Finally, two reports (Nieman et al., 2013, Stracke et al., 2010) were excluded as phenolic acids were reported as relative concentrations rather than absolute (Figure 5.1).

A total of 79 published studies satisfied the inclusion criteria and reported plasma levels of polyphenol and polyphenol metabolites following a dietary intervention. From those, 9 reports focused on longer-term feeding studies while the rest covered acute changes in polyphenol levels following a single consumption of a test food (Figure 5.1). Acute trials mainly focused on polyphenol bioavailability in the 5-24 hour window following ingestion, while longer-term feeding studies investigated changes in polyphenol metabolites levels after 5 days to 8 weeks supplementations (Grimm et al., 2006, Koli et al., 2010) (Table 5.1).

Most studies (Table 5.1) focused on a single food product, from a diverse range including tea (black and green) (Henning et al., 2013), coffee (Kempf et al., 2010), olive oil (Oliveras-Lopez et al., 2012), cocoa (Urpi-Sarda et al., 2009) and berries, either as a juice (Karlsen et al., 2010) or a mixed berry diet (Koli et al., 2010). Two studies used extracts instead of a food product (Boyle et al., 2000, Grimm et al., 2006). Regarding sample treatment prior to phenolic acids measurements, only two of the studies measured free phenolic acids (i.e. without employing a prior hydrolysis step to derive aglycones from glucuronide and sulfate esters) (Heinrich et al., 2013, Oliveras-Lopez et al., 2012). One of these two studies did not detect sufficient phenolic acids in plasma for quantification, despite using a HPLC/MS (LCQ Fleet quadripole ion-trap MS) detection system (Heinrich et al., 2013), and the other focused on hydroxytyrosol as a marker of compliance (olive oil intake) (Oliveras-Lopez et al., 2012).

Figure 5.8 Flow diagram of the study selection process
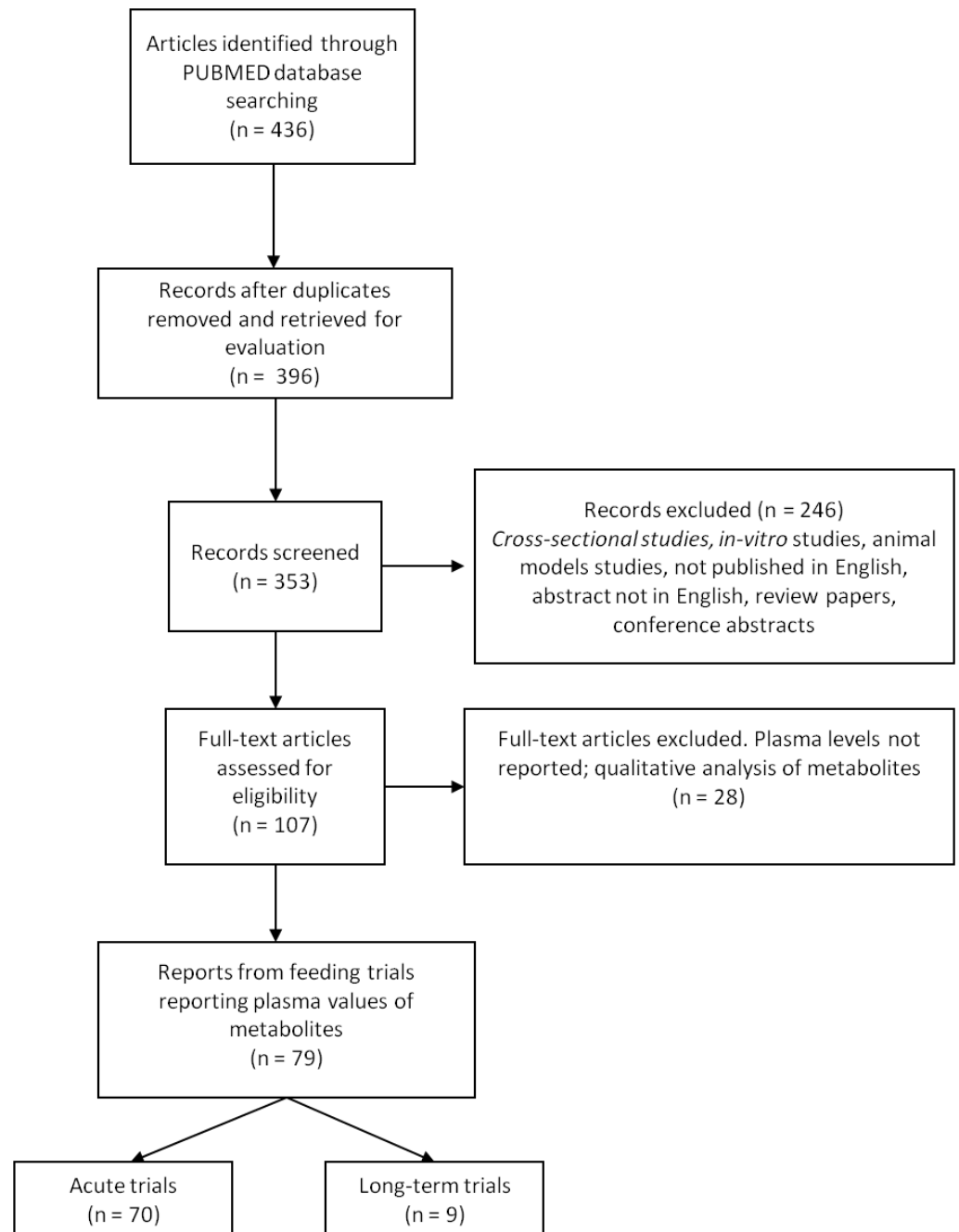
Table 5.1 Evidence table of long-term supplementation trials measuring phenolic acids in plasma.

Authors	Food	Study design	Duration	Metabolites	Hydrolysis	Comments
Heinrich et al. (2013)	Blue honeysuckle berry (165g/d)	Uncontrolled (n=10)	1 wk	Below limit	No	Low polyphenol diet throughout
Henning et al. (2013)	Black tea (6 cups/d)	Non-blinded RCT, parallel (n=46)	3-6 wks	**3-hydroxyphenylacetic acid:** 261μM **4- hydroxyphenylacetic acid:** 668μM **3,4-dihydroxyphenylacetic acid:** 117μM **Hippuric acid:** 2305μM **Homovanillic acid:** 176μM	Yes	No dietary info
Henning et al. (2013)	Green tea (6 cups/d)	Non-blinded RCT, parallel (n=47)	3-6 wks	**3- hydroxyphenylacetic acid:** 338μM **4- hydroxyphenylacetic acid:** 798μM **3,4-di hydroxyphenylacetic acid:** 135μM **Hippuric acid:** 1950μM **Homovanillic acid:** 199μM	Yes	No dietary info
Oliveras-Lopez et al. (2012)	Extra virgin olive oil as a fat replacement plus 50mL raw	Non-blinded cross over (n=20)	4 wks	**Hydroxytyrosol:** 487nM	No	Habitual diet as control
Karlsen et al. (2010)	Bilberry juice (1L diluted in water)	Non-blinded parallel RCT (n=63)	4 wks	**Quercetin:** 43.6nM **m-coumaric:** 12.8nM **p-coumaric:** 27.4nM **protocatechuic:** 99.4nM	Yes	3wks low antioxidant/berry diet

| **Kempf et al. (2010)** | Coffee 4 or 8 cups/d (150mL) | Single blind cross over (n=47) | 30 d | **4cups**
Caffeic acid: 38.3µM
Dihydrocaffeic acid: 47.9nM
m-Coumaric acid: 26.4nM
Dihydro-3-coumaric: 716nM
Ferulic: 55.1nM
Isoferulic: 23.5nM
Dihydroferulic: 93.9nM
Dihydroisoferulic: 56.5nM
Dimethoxycinnamic: 77 nM
3-(3,4-Dimethoxyphenyl)-propionic: 203nM
8cups
Caffeic acid: 62.2µM
Dihydrocaffeic acid: 75.2nM
m-Coumaric acid: 58.8nM
Dihydro-3-coumaric: 1583nM
Ferulic: 67.1nM
Isoferulic: 49.8nM
Dihydroferulic: 194.7nM
Dihydroisoferulic: 90.6nM
Dimethoxycinnamic: 177.7nM
3-(3,4-Dimethoxyphenyl)-propionic: 398nM | Yes | No wash out, All subjects consumed 0 cups (1st month), 4 cups (2nd month), 8 cups (3rd month) |
| **Koli et al. (2010)** | 100g bilberries and nectar containing 50g lingonberries/ 100g black-currant-strawberry puree (80% black currant) | Non blinded RCT, parallel (n=72) | 8 wks | **Quercetin:** 40nM
Caffeic acid: 100nM
Protocatechuic acid: 120nM
p-coumaric: 15nM
Vanillic: 70nM
3-(3-hydroxyphenyl)-propionic: 800nM
3-Hydroxyphenylacetic: 275nM
Homovanillic: 90nM
3,4-dihydroxyphenylacetic acid: 140nM | Yes | The two supplements were consumed on an alternate day basis |

Urpi-Sarda et al. (2009)	Cocoa powder 40g/d with 500mL skimmed milk	Non-blinded RCT Cross-over (n=42)	4 wks	**3,4-Dihydroxyphenylpropionic acid:** 0.2µM **3-Hydroxyphenylpropionic acid:** 0.23 µM **3,4-Dihydroxyphenylacetic acid:** 0.11µM **3-hydroxyphenylacetic acid:** 0.12 µM **Phenylacetic acid:** 20.32 µM **p-coumaric acid:** 0.03 µM **caffeic acid:** 0.08µM **Ferulic acid:** 0.21 µM **Protocatechuic acid:** 10.52µM **Vanillic acid:** 2.71µM **4-hydroxybenzoic acid:** 9.73µM **4-Hydroxyhippuric acid:** 0.11µM **3-Hydroxyhippuric acid:** 0.48µM	Yes	No wash-out
Grimm et al. (2006)	Pine bark extract (200mg/d)	Non-controlled (n=5)	5 d	**Catechin:** 170nM **Caffeic acid:** 13.4nM **Ferulic acid:** 103nM	Yes	Measured 4h after the last dose
Boyle et al. (2000)	Rutin supplement (500mg/d)	Double blind RCT parallel (n=16)	6 wks	**Quercetin:** 166nM **Kaempferol:** 5.24nM **Isorhamnetin:** 9.49nM	Yes	Record flavonoid rich food

The major phenolic acids identified in the studies were: 3-hydroxyphenylacetic acid (120nM-338µM), 3,4-dihydroxyphenylacetic acid (110nM-135µM), homovanillic acid (90nM-199µM), m-coumaric acid (12.8-58.8nM), p-coumaric acid (15-30nM), caffeic acid (13.4nM-62.2µM), protocatechuic acid (99.4nM-10.52µM), ferulic acid (55.1-210nM) and vanillic acid (70nM-2.71µM). The compounds measured and identified all represent concentrations of aglycones after hydrolysis and not concentrations of free compounds.

A combination of 3-hydroxyphenylacetic acid (160nM), 3,4-dihydroxyphenylacetic acid (40nM), vanillic acid (20nM), p-coumaric acid (10nM), caffeic acid (10nM) and protocatechuic acid (40nM) was selected for the *in-vitro* glycation of BSA. These phenolic acids represent only a selection of all the acids identified in plasma after feeding interventions, but they are the ones for which data on absorption and metabolism are more extensive. The glucuronidation of polyphenols and phenolic acids may have a negative effect on the antiglycative capacity of the molecules (Xie and Chen, 2013). These concentrations take into consideration the fact that, phenolic acid levels in all the studies in the literature review were measured after hydrolysis, and so a factor was applied to estimate what percentage of the reported values would be non-conjugated phenolic acids.

This percentage was calculated based on findings from previous studies and more specifically, data from acute feeding interventions identified during the process of the present literature review. From acute feeding interventions measuring both the conjugated and non-conjugated form of phenolic acids, only 20-26% of the total caffeic found in plasma after coffee consumption is in free form (Nardini et al., 2002, Nardini et al., 2009). Similarly only 25% of the total vanillic acid, 40-50% of the total p-coumaric acid and 15-25% of the total ferulic acid are found as free phenolic acids(Nardini et al., 2009, Nardini et al., 2006). In the contrary 4-hydroxyphenylacetic acid is less extensively metabolised (80% present as free form) (Nardini et al., 2006).

The published reports were of variable methodological quality. Although the detection systems used for phenolic acids measurement were based on mass spectrometry (MS, MS/MS, quadripole MS), some studies used less sensitive detection systems like ultraviolet, fluorimetric and electrochemical detectors(Kempf et al., 2010, Grimm et al., 2006, Boyle et al., 2000). One of the major methodological limitations was that 7 out of 9 studies employed some form of hydrolysis prior to phenolic acids determination. The measurement of aglycones following hydrolysis leads to a significant increase in the concentrations reported. The only study measuring phenolic acid concentrations without hydrolysis did not detect concentrations high enough for quantification even with a sensitive quadripole MS system (Heinrich et al., 2013).

The variable duration and dose of the compounds tested also make comparisons between the studies available difficult. The compounds tested were delivered in various matrices, some as supplements (Grimm et al., 2006, Boyle et al., 2000), others as drinks (Henning et al., 2013, Kempf et al., 2010, Karlsen et al., 2010, Urpi-Sarda et al., 2009), or whole foods (Heinrich et al., 2013) and even as dietary pattern (combination of various food items) (Koli et al., 2010). Such matrices will have a significant effect on bioavailabilty and potential long-term changes in absorption through changes in gut microbiota cannot be excluded/ controlled for. The lack of successful control conditions is another point to be mentioned, as some studies either had no control groups or used a cross-over design without allowing for sufficient wash-out periods (Kempf et al., 2010, Urpi-Sarda et al., 2009, Grimm et al., 2006). When bioavailability of (poly)phenolic compounds is the main focus of the study the lack of a control group may be of lesser importance but not allowing for sufficient wash-out periods can introduce a carry-over effect between the interventions making difficult to compare between doses/groups tested.

Finally, as (poly)phenolic compounds are nearly ubiquitous, controlling for the effect of the background diet is important when studying bioavailability. In the studies reviewed, background diet control was variable ranging from the subjects being requested to abstain from polyphenol sources throughout the experimental period (Heinrich et al., 2013, Karlsen et al., 2010) to being asked to consume their habitual diet (Oliveras-Lopez et al., 2012). One study requested from the participants to avoid food products similar to those provided during the study period (Koli et al., 2010) and another gave no advice but requested from the participants to keep records of the flavonoid rich foods consumed during the study (Boyle et al., 2000). In order to control for the effect of background diet, Grimm et al requested from their volunteers to follow a flavonoid free diet 24 hours prior to blood sampling (Grimm et al., 2006). Unfortunately three of the studies did not provide with any information on whether and how they attempted to control for the effect of the background diet (Henning et al., 2013, Urpi-Sarda et al., 2009, Kempf et al., 2010). In this instance lack of adequate control of the background diet was not considered as a bias given the purpose of the review.

In-vitro assessment of antiglycative capacity of phenolic acids

The antiglycative capacity of phenolic acids was investigated in a two dimensional design. Phenolic acids were tested for their capacity to reduce fructosamine production when i) added in the reaction solution alongside glucose and H_2O_2 and ii) when pre-incubated with albumin (BSA) prior to the glycation incubation.

Table 5.2 Fructosamine concentration (mM DMF equivalent) after two week incubation with hydrogen peroxide or phenolic acids.

Glucose level (mM)	Pre-treatment		
	Native Mean (SD)	Pre-oxidised Mean (SD)	Phenolic-rich Mean (SD)
0	0.22 (0.01)	0.23 (0.02)	0.23 (0.02)
5	0.29 (0.01)	0.32 (0.01)	0.32 (0.02)
10	0.36 (0.02)	0.41 (0.03)*	0.40 (0.03)*
0+ H_2O_2 (10nM)	0.19 (0.04)	0.20 (0.02)	0.23 (0.01)
5+ H_2O_2 (10nM)	0.32 (0.03)	0.31 (0.03)	0.28 (0.02)*
10+ H_2O_2 (10nM)	0.40 (0.03)	0.40 (0.04)	0.31 (0.03)*
0+Phenolic acids	0.22 (0.01)	0.22 (0.02)	0.22 (0.01)
5+Phenolic acids	0.31 (0.02)	0.30 (0.02)	0.29 (0.02)
10+Phenolic acids	0.38 (0.04)	0.38 (0.03)	0.36 (0.03)

*$p < 0.05$ vs native

Effect of addition of hydrogen peroxide and phenolic acids in the reaction solution

Addition of 10nM H_2O_2 in the reaction solution throughout the incubation has a significant but opposing impact on fructosamine production of both native and phenolic pre-incubated albumin. Incubation of native albumin in the presence of H_2O_2 led to significantly higher levels of fructosamine at 10mM glucose compared to the glucose-only control (14% increase), whereas a reduction in fructosamine production was seen in the phenolic pre-incubated albumin (Table 5.2). A two-way ANOVA analysis showed that H_2O_2 affects fructosamine production of native and phenolic pre-incubated albumin independently of glucose ($p < 0.001$ for both albumin forms; H_2O_2 plus glucose vs. glucose only control).

On the contrary addition of phenolic acids in the reaction solution throughout the incubation period had no significant effect on fructosamine production compared to glucose alone in any of the three albumin forms used (Table 5.2).

Effect of protein pre-treatment

Pre-oxidised and phenolic-preincubated albumin was more prone to glycation than the native molecule in the presence of 10mM glucose (p=0.001 and p=0.02, respectively) (Table 5.2). This effect was independent of glucose concentration, for both the pre-oxidised and the phenolic-preincubated albumin (p=0.001, two-way ANOVA). In the presence of H_2O_2, native and pre-oxidised albumin showed similar glycation levels (p=0.52; two-way ANOVA).

Preincubation with phenolic acids, on the other hand, significantly reduced glycation in the presence of H_2O_2 (p=0.001 vs native; two-way ANOVA). This effect was seen at both 5 and 10mM glucose (p=0.01 and p<0.001, respectively, vs. native albumin) (Figure 5.3) and the glycation reduction was greater with increasing glucose levels (p<0.001 for the interaction pre-treatment*glucose levels).

Figure 5.9 Fructosamine concentration (mM DMF equivalent) after two weeks incubation in the presence of glucose and H_2O_2 (10nM) for native, pre-oxidised and phenolic-preincubated BSA (PP-BSA).
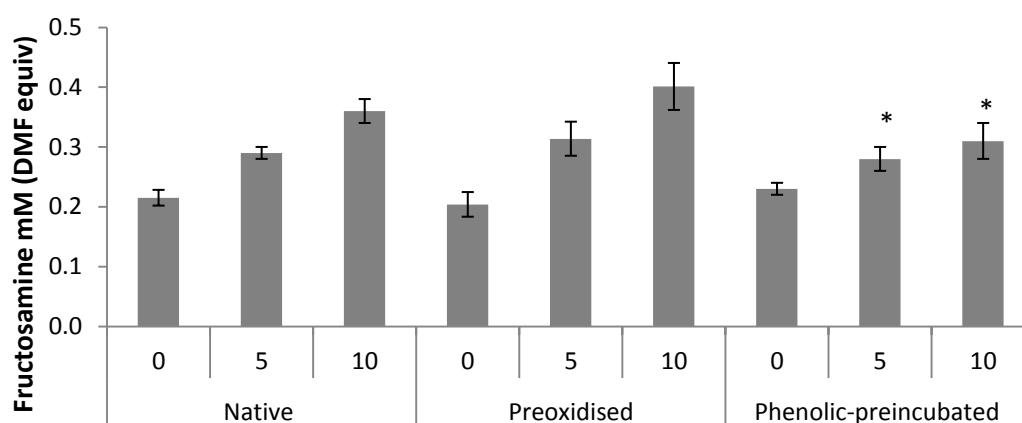
Two-way ANOVA analysis showed a significant effect of phenolic-preincubation inhibiting glycation. *p<0.05

Effect on AGEs production

After two weeks incubation, AGEs levels were not higher in any experimental condition compared to the 0mM glucose control, but for oxidised BSA exposed to 10mM glucose with 10nM H_2O_2 (p =0.048, 205±15.8 vs.284±76.1 AU ; oxBSA 0mM glucose plus 10nM H_2O_2 vs. oxBSA 10mM glucose plus 10nM H_2O_2).

Effect of protein pre-treatment on protein structure and characteristics

The circular dichroism (CD) analysis showed no effect of the pre-treatment on the secondary protein structure (Figures 5.11 & 5.12). However, exposure to 10nM H_2O_2 for 8hrs lead to a 25% decrease in tryptophan fluorescence. Pre-incubation with phenolic acids for 8hrs resulted to a 38% reduction, indicative of protein-phenolic acid binding (Figure 5.10).

Figure 5.10 Emission spectra of 0.2g/L BSA, ox-BSA and PP-BSA at λ=277nm showing the quenching effect of protein pre-treatment. Spectra were recorded at pH 7.4.
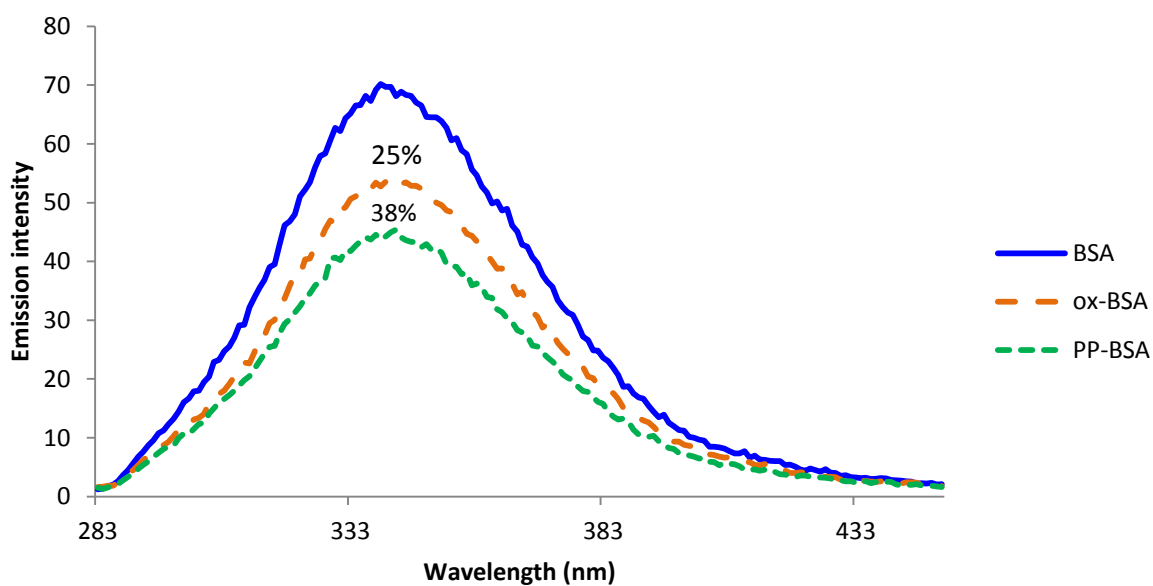
Figure 5.11 Near UV Circular Dichroism spectra of 1.5mg/ml of native BSA (blue), oxidised BSA (green) and phenolic-treated BSA (red). Spectra were recorded in a 0.5cm pathlength quartz cuvette using a Jasco J-810 spectropolarimeter.
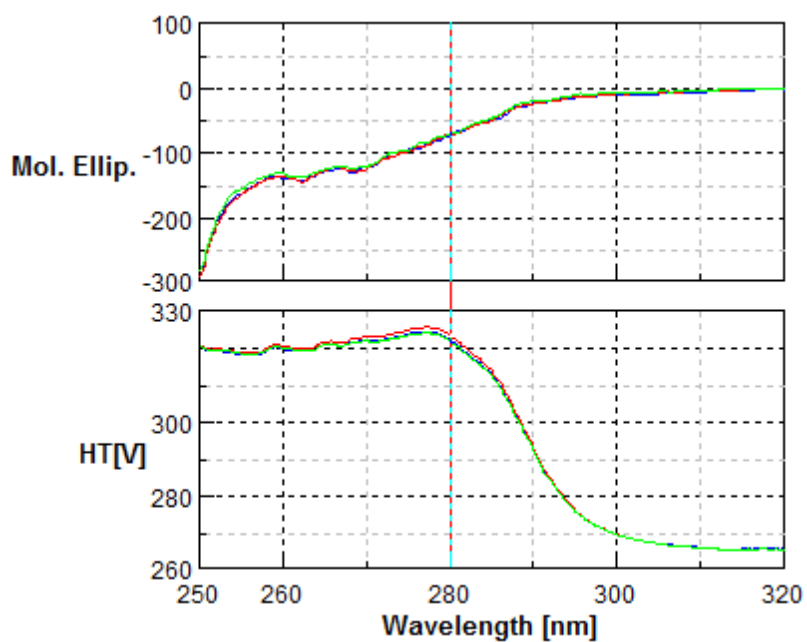
Figure 5.12 Far UV Circular Dichroism spectra of 0.2mg/ml of native BSA (blue), oxidised BSA (green) and phenolic-treated BSA (red). Spectra were recorded in a 0.02cm pathlength quartz cuvette using a Jasco J-810 spectropolarimeter.
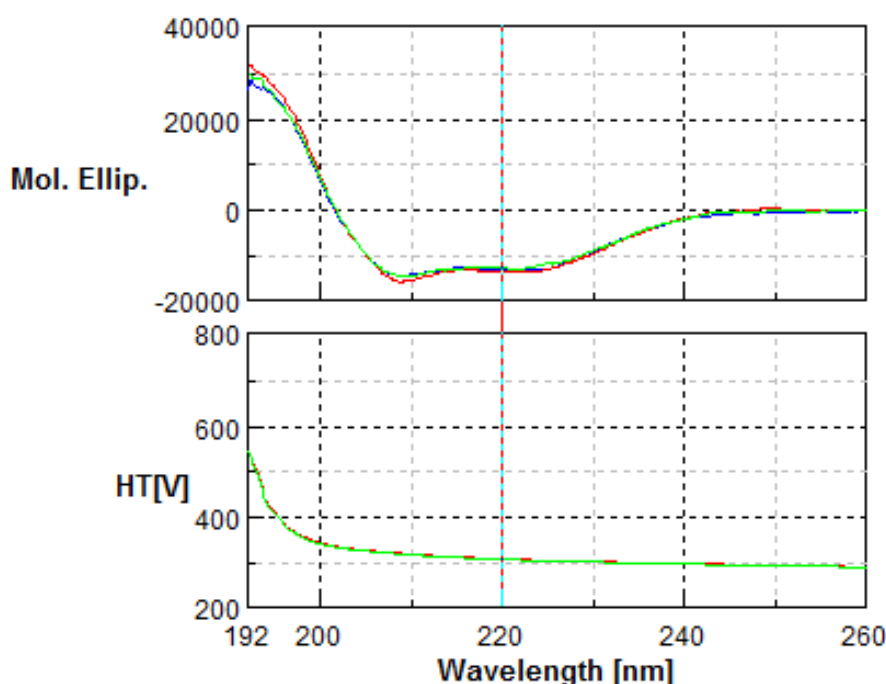
5.4 Discussion

Our mechanistic study results indicate that pre-treatment of albumin with phenolic acids inhibits fructosamine production, especially in the presence of oxidative stress or oxidative damage. This antiglycative activity was apparent when comparing the glycation achieved using phenolic-enriched albumin to those with the native and pre-oxidised BSA. A two-way ANOVA analysis showed that, in the presence of H_2O_2, albumin pre-incubated with phenolic acids had a significantly lower fructosamine content compared to the native BSA and the pre-oxidised BSA molecule. Phenolic acid preincubation only offered protection against fructosamine production in the presence of H_2O_2 10nM: it provided no protection against glycation by glucose alone. In the presence of H_2O_2, the antiglycative activity of phenolic acid pre-incubation was greater with higher glucose levels (10% vs. 22.5% decrease at 5 and 10mM glucose respectively). No effect was seen for AGEs production, with the exception of pre-oxidised BSA exposed to a combination of 10nM H_2O_2 and 10mM glucose. This maybe due to the duration of the experiment, too short to lead to AGEs formation in the given glucose concentrations.

In contrast with most of the literature to date, which suggests that polyphenols and phenolic acids added to the incubation solution provide potent antiglycative activity (Lunceford and Gugliucci, 2005, Kim and Kim, 2003, Bousova et al., 2005, Wu and Yen, 2005, Nagasawa et al., 2003), our results show this to be unlikely. In the previous investigations, concentrations used were non-physiological, with the lowest glucose concentration being 30mM (>5-6 fold higher than normoglycaemia), generating higher glycation than our use of physiologically relevant 5 and 10 mM concentrations, representative of normoglycaemic and diabetic conditions respectively (Vlassopoulos et al., 2013b). Our results suggest that pre-incubation of albumin with phenolic acids is the most likely mechanism to offer protection against glycation in a physiologically relevant system. Pre-incubation of BSA with either 10nM H_2O_2 or phenolic acids did not affect the secondary structure of the molecule but both led to the reduction of tryptophan fluorescence. Tryptophan is an established oxidation target in the BSA molecule (Guedes et al., 2009), and oxidation would modify its fluorescence. The BSA molecule has only two tryptophans: one inside a α-helix in domain I and another one inside the hydrophobic binding pocket of domain II (Majorek et al., 2012, Tayeh et al., 2009). A proposed mechanism of action from our result involves steric hindrance, with phenolic acids binding in a BSA locum which includes a tryptophan and consequently preventing the amino acids in this locum are to participate in oxidation and subsequent glycation reactions. (since BSA oxidation increases its susceptibility to glycation (Vlassopoulos et al., 2013).

The literature review highlighted that 3-hydroxyphenylacetic acid, 3,4-dihydroxyphenylacetic acid, homovanillic acid, m-coumaric acid, p-coumaric acid, caffeic acid, protocatechuic acid, ferulic acid and vanillic acid are most commonly detected in plasma after long-term feeding interventions. Concentrations of phenolic acids ranged between 13.4nM-338.0µM. The majority of the studies measured phenolic acid levels as aglycones after hydrolysis of conjugated phenolic acids. This could have led towards a systematic overestimation of phenolic acid levels. Phenolic acids are subject to extensive rapid metabolism (Scalbert et al., 2002, Scalbert and Williamson, 2000) and since the chemical properties of their conjugates are often different, even opposite, from those of the aglycones (Xie and Chen, 2013) it is important for *in-vitro* studies to take their relative abundance into consideration. More *in-vivo* studies, reporting phenolic acid levels as aglycones and conjugates separately, are needed.

Nonetheless, the results of the literature review showcase that the use of food extracts or food-derived polyphenol molecules in mechanistic studies focusing on human metabolism is likely to be of limited physiological relevance. The parent compounds commonly used for *in vitro* studies are rarely found in circulation, while metabolites like phenolic acids are found in potentially important concentrations in the nM to µM range. These metabolites are also not seen in isolation, but in

fairly consistent combinations of phenylacetic acids, caffeic acid, vanillic acid and protocatechuic acid.

Despite increasing evidence that a very limited fraction of dietary polyphenols are absorbed, with low levels of metabolites circulating for limited amount of time, *in-vitro* models to-date utilise designs that make them irrelevant to physiology outside the gut lumen (Daiponmak et al., 2014, Harris et al., 2014, Ito et al., 2014, Dorsey and Greenspan, 2014). Most of these reports used supra-physiological glucose and albumin concentrations (Daiponmak et al., 2014, Dorsey and Greenspan, 2014, Harris et al., 2014) as well as methanolic extracts of food products (Ho et al., 2013, Cervato et al., 2000, Hegde et al., 2002). While of importance for the food industry (Zhang et al., 2014), they hold limited translational value for human health and may confuse our understanding of the role of glycation in health and disease. Here, a combination of caffeic acid, p-coumaric acid, vanillic acid, protocatechuic acid, 3-hydroxyphenyl acetic acid, and 3,4-dihydroxyphenyl acetic acid was chosen to replicate physiological conditions, according to the systematic review.

This study has several limitations. The *in-vitro* design is in itself limiting and translation to physiology must still be cautious. Concentrations were chosen to mimic physiology for glucose, H_2O_2, and phenolic acids but this made it impossible to draw conclusions on the effect of individual phenolic acid on glycation. Not all our results fit a simple mechanistic explanation. It is intriguing that our results suggest that phenolic-preincubated albumin was more prone to glycation than native albumin, in the presence of 10mM glucose. We can offer no plausible mechanism to explain this, which may have been a random or chance effect. Nonetheless, this study has provided evidence to extend the published literature, showing that research into the antiglycative capacity of polyphenols is still possible at physiologically relevant concentrations of phenolic acids, and at physiological glucose concentrations, much lower than have been shown to generate glycation previously.

5.5 Conclusion

Phenolic acids have the capacity to modulate early stages of protein glycation under normoglycaemic and at physiogical concentrations. Incubation with phenolic acids prior to glycation significantly inhibits the process in the presence of oxidative stress. Designing *in-vitro* studies with a high degree of physiological relevance is very important in order to reach biologically sound conclusions.

Chapter 6: Polyphenol intake and maternal glycation during pregnancy. Maternal diet and glycoxidation as risk factors for complications and neonatal anthropometry in obese pregnancies

Abstract

Background: Improved redox status and polyphenols have been shown to reduce glycation *in-vitro*. In pregnancy, maternal glycation and sRAGE levels can be used as diagnostic markers for complications and may be relevant to fetal programming. The association between diet and glycation in pregnancy and their effect on maternal and neonatal health are still to be explored.

Methods: Samples and data from the UPBEAT study trial (n=117) were analysed. Plasma fructosamine, plasma sRAGE, urinary Ferric Reducing Ability of Plasma (FRAP), urinary Total Phenol (TP)and urinary Advanced Oxidised Protein Products (AOPPs) were measured at $16\text{-}18^{+6}$ and $27\text{-}28^{+6}$ weeks gestation. Dietary recalls were used to calculate fruit and vegetable and polyphenol intake at the same timepoints. Data were analysed to identify associations between dietary variables and biochemical markers, as well as their relationships with diagnosis of complications. Associations between maternal variables and neonatal anthropometry were also investigated.

Results: Fructosamine and AOPPs fell by 3 and 22%, respectively between $16\text{-}18^{+6}$ and $27\text{-}28^{+6}$ weeks gestation. Women in the lowest quartile of total polyphenol intake had 8% greater fructosamine levels compared to those in the top quartile. Total polyphenol intake was negatively correlated with sRAGE levels. Diagnosis of severe preeclampsia was associated with elevated AOPPs. Maternal polyphenol intake was positively correlated with birth weight, while maternal glycoxidation showed the opposite relationship.

Conclusion: This study is the first to demonstrate an association between polyphenol intake and glycation during pregnancy, with an impact on neonatal outcome measures. Maternal glycoxidation is a promising marker of pre-eclampsia and neonatal anthropometry.

6.1 Introduction

Nearly a third of all women are overweight or obese at the time of conception, with the prevalence of BMI≥30 during pregnancy reaching 20% (Kim et al., 2007, Huda et al., 2010, Heslehurst et al., 2010, Heslehurst et al., 2007). Increased adiposity during pregnancy is associated with higher risk of developing gestational diabetes and preeclampsia (Huda et al., 2010, Baeten et al., 2001, Heslehurst et al., 2008). Moreover, nearly 50% of obese pregnant women will require assisted delivery, caesarean section or instrumental delivery (Weiss et al., 2004, Chu et al., 2007, Leung et al., 2008, Janga et al., 2011, Sydsjo et al., 2010), alongside a generally increased length of hospital stay (Mamun et al., 2011, Galtier-Dereure et al., 2000).

The increased health risk from obesity during pregnancy does not only affect the mother, but it has a substantial impact on the fetus, both *in utero* (stillbirth, congenital anomalies) and during the neonatal period (Heerwagen et al., 2010, Tenenbaum-Gavish and Hod, 2013, Leddy et al., 2008). Children born to obese mothers are more likely to be born large for gestational age (Mutsaerts et al., 2014, Oza-Frank and Keim, 2013, Li et al., 2013, Rasmussen et al., 2014, Heslehurst et al., 2008, Ray et al., 2001). They are also more likely to be born with insulin resistance (Catalano et al., 2009), to present with meconium aspiration (Magann et al., 2013) and to require medical attention in the first days of life (Minsart et al., 2013, Aimukhametova et al., 2012, Ray et al., 2001).

Inflammation and protein glycation have been proposed as candidate diagnostic markers of obesity-related complications during pregnancy (Romero et al., 2007, Haedersdal et al., 2013, Nicholson et al., 2013, Sado et al., 2011, Harsem et al., 2008). Glycation is the non-enzymatic condensation of a sugar moiety on protein/lipid molecule (Maillard, 1912, Hodge, 1953), and although it has been established as a marker of disease pathogenesis in diabetes (Sabanayagam et al., 2009, Yan et al., 2003), it only recently attracted attention in the context of normoglycaemia, with both epidemiological and mechanistic data to support a positive impact of oxidative stress on physiological protein glycation (Selvin et al., 2005, Selvin et al., 2010, Vlassopoulos et al., 2013a, Vlassopoulos et al., 2013b). A systematic review of the literature showed a positive relationship between glycation, measured as HbA1c, and cardiovascular disease risk for non-diabetic individuals (see Chapter1).

In pregnancy, conditions like PCOS and diabetes, both associated with higher glycation levels, have also been associated with higher risk of complications (Roos et al., 2011). Maternal glycation levels correlate strongly with IVF success and neonatal glycation levels at birth and 6 months after birth (Jinno et al., 2011, Boutzios et al., 2013). Activation of RAGE has been proposed as a

pathway leading to the development of preeclampsia both through increased concentrations of RAGE ligands and higher expression of the RAGE gene (Naruse et al., 2012, Cooke et al., 2003). High levels of sRAGE are associated with lower likelihood of success following assisted reproduction and low quality of embryonic development (Malickova et al., 2010, Bonetti et al., 2013). During the course of a healthy pregnancy, sRAGe levels are generally decreased compared to non-pregnant controls, but sRAGE values are not static as they peak during the 2nd trimester compared to 1st and 3rd trimester (Germanova et al., 2010). Changes in sRAGE levels may have a predictive values in terms of pregnancy complications as they seem to be consistently increased in women who developed preeclampsia (Germanova et al., 2010, Oliver et al., 2011, Kwon et al., 2011) and decreased in women who experienced preterm labour (Bastek et al., 2012, Germanova et al., 2010).

AGEs are implicated in disease pathogenesis through RAGE mediated action (Yan et al., 2003, Jandeleit-Dahm et al., 2008). RAGE is a multi-ligand cell-surface immunoglobulin, with the ability to initiate injury-like intracellular events, mainly expression of genes related to inflammation and oxidative stress (Schmidt et al., 1995, Hofmann et al., 1999, Kislinger et al., 1999). When activated by AGEs, RAGE can lead to increased ROS production and higher inflammation (IL-6, TNF-α) via NfκB activation (Coughlan et al., 2009, Vazzana et al., 2009, Schmidt et al., 2000). sRAGE, a molecule homologous to RAGE (Vazzana et al., 2009, Chen et al., 2014, Maillard-Lefebvre et al., 2009, Raucci et al., 2008), has the capacity to bind on AGEs and act as a scavenger not allowing circulating AGEs to bind on RAGE (Raucci et al., 2008, Lindsey et al., 2009, Koyama et al., 2007, Maillard-Lefebvre et al., 2009).

Lifestyle and diet can have a significant impact on glycation levels in normoglycaemia. Higher intake of vitamins C and E, and higher vegetable intake, have all been associated with lower levels of HbA1c in normoglycaemic subjects (Bates et al., 2004, Samaha et al., 2003, Sargeant et al., 2000, Vlassopoulos et al., 2013a, Boeing et al., 2000). Similarly, polyphenol supplementation may lead to a reduction in HbA1c levels but the evidence is still inconclusive. Conversely, higher saturated fat intake and smoking have been related with higher HbA1c levels (Harding et al., 2001, Vlassopoulos et al., 2013a, Clair et al., 2011), while intakes of carbohydrates, protein, fibre and lower physical have no effect on glycation levels (Boeing et al., 2000, Sahyoun et al., 2005).

The UPBEAT trial is a randomised controlled multi-centre nutritional intervention. Participants in the intervention arm of UPBEAT were offered an 8-week intervention aiming to reduce the dietary glycaemic index (GI) and increase physical activity, to be compared with standard care. The reduction in glycaemic index was to be achieved by higher consumption of low glycaemic index foods and replacement of sugar-sweetened beverages with low GI alternatives. Alongside, GI reduction women in the intervention arm were asked to replace saturated fats with mono and

polyunsaturated fats (MUFA, PUFA). As low glycaemic food products like wholemeal cereal, vegetables and legumes are also high in antioxidant compounds (Ludwig, 2002, Slavin, 2003, Hu, 2003, Jenkins et al., 2002) we hypothesised that women in the intervention arm could benefit from an improved redox status, compared to the control. With the potential to reduce saturated fat intake and increase the intake of fruit, vegetables and antioxidant compounds, the dietary intervention offered through the UPBEAT trial could have an impact on glycation markers.

So far, little is known about how nutrition and other lifestyle factors can impact on glycation and sRAGE levels of pregnant women. Even less evidence is available to link dietary habits, biochemical parameters and obesity-related complication during pregnancy. This study aimed to assess the following research questions: 1) Whether dietary fruit and vegetable, as well as antioxidant intake, in obese pregnant women modulate glycation in the mother and 2) whether maternal glycation and sRAGE levels are associated with pregnancy complications and newborn anthropometry.

6.2 Methods

Study design and participants

Samples and data were obtained from the UPBEAT pilot study. UPBEAT is a RCT studying the effect of a lifestyle intervention aimed to reduce glycemic index on pregnancy complications and neonatal health. The full protocol with details has been published elsewhere (Briley et al., 2014, Poston et al., 2013). In summary, women with a BMI ≥30 kg/m^2 and a singleton pregnancy were recruited during the 15-17^{+6} weeks gestation and randomised to either the control (n=59) or intervention group (n=58). Potentially eligible participants attending clinics for general antenatal care were approached by research midwives in four UK study centres in urban settings (the Southern General Hospital and Princess Royal Maternity Hospital, Glasgow; the Royal Victoria Infirmary, Newcastle; Guys' and St Thomas' NHS Foundation Trust and King's College Hospital Foundation Trust, London). The intervention group received an 8-week long training, based on control theory with elements of social cognitive theory. Participants attended weekly group sessions delivered by trained midwives, from approximately 19 weeks gestation. Using an exchange system, women were recommended to increase consumption of foods with low dietary GI and to reduce consumption of sugar sweetened beverages (primary goal), and to reduce saturated fat intake. The aim was for the dietary changes to be achieved in an isocaloric manner, so women were given information on how to exchange high GI food items for low GI equivalents without reducing their total energy intake. Women in the intervention group were also prompted to monitor their physical activity and set goals in order to increase it. Participants were asked to

maintain the changes achieved during the intervention period for the remainder of the pregnancy. Women in the control group were recruited at the same time-point and asked to revisit at 27-28^{+6} weeks gestation. This report used samples and data from the UPBEAT study pilot (n=108), which were available at the time of the analysis. The full trial is currently ongoing with an anticipated sample size of 1,546 participants by the end of April 2014.

Maternal pregnancy and newborn outcome data

Data recorded by the UPBEAT team as part of routine clinical practice included diagnosis of preeclampsia, gestational diabetes, gestational age at delivery and mode of delivery among others. Newborn variables included birthweight, customised birthweight centile and neonatal anthropometry. Newborn data used for the current analysis focused on the newborn being characterised as small for gestational age (SGA) or macrosomic/ large for gestational age (LGA). Customised birthweight centiles were calculated correcting for gestational age, maternal ethnicity, weight and height in early pregnancy, parity and infant sex. LGA was defined >90th customised birthweight centile; SGA as ≤10th customised birthweight centile.

Dietary Assessment

Dietary assessment was performed by research midwives trained in dietary assessment techniques, using a triple pass 24-hour dietary recall method and a short FFQ to determine dietary intake at baseline, post-intervention and follow-up visits. Dietary coding was undertaken by a research dietician and entered into the study database using McCance and Widdowson "Composition of Foods" (6th edition) food codes. The nutritional analysis software WISP 3.0 (Tinuviel Software) was used to evaluate nutrient composition of the diet. Values for the 24 hour recall data which were obtained twice for each gestational period (one week apart) were averaged. When participants provided one recall per timepoint, this was treated as the representative intake at that timepoint.

Fruit & vegetable consumption

Intake of fruit and vegetables in portions per day was calculated directly from the dietary recalls. Fruit and vegetable consumption was calculated as consumption of whole fruit and whole vegetable as well as fruit and vegetable composites. Fruit composites included any dish that was based on fruit products or included fruits. Consumption of jams, juices, smoothies, tarts, fruit cakes etc. was analysed as fruit composites. Similarly all dishes containing vegetable and/or vegetable compotes/pastes were analysed as vegetable composites. Bouillons and vegetable stocks were not included in the analysis. Food products containing both fruit and vegetables were analysed under both categories respectively. A representative sample of minimum three recipes

was used to calculate the proportion corresponding to fruit/vegetable per recipe. Percentages were calculated based on uncooked net weight with added water if provided in the recipe. Fruit and vegetable intake was calculated as 80g portions or 120mL portions for juices.

Polyphenol intake

Total polyphenol intake at each timepoint was calculated, based specifically on fruit, vegetable, vegetable composite and fruit composite consumption, plus food items rich in polyphenols like wines, teas, coffees, infusions, cocoa products and spices. For each food item, a total polyphenol value per 100g was assigned based on data obtained through the PhenolExplorer database (Rothwell et al., 2012). Analysis was only performed as total polyphenol intake and not as intake of polyphenol subclasses (eg flavonoids, phenolic acids etc.)

Lifestyle and socio-demographic data; anthropometry; blood and urine sample collection

At the randomisation visit, participants completed a series of questionnaires assessing socioeconomic status, living arrangements, employment etc. as well as lifestyle factors including smoking and number of cigarettes smoked per day among other variables of interest. Weight, height and waist circumference were measured using standard protocols. Blood and spot urine samples were collected following an overnight fast at each timepoint. Blood and urine samples were aliquoted and stored at -80°C.

Laboratory analysis

Fructosamine values were measured on a clinically validated platform (c311, Roche Diagniostics, Burgess Hill, UK) in using the manufacturers' quality controls and calibration materials. Levels of plasma sRAGE were measured using an enzyme linked immunosorbent assay (ELISA) using the manufacturers calibrators and protocols (RayBioTech Inc., Norcross, Georgia, USA). Urinary levels of Total Phenol (TP) and Ferric Reducing Ability of Plasma (FRAP) and Advanced Oxidation Protein Products (AOPPs) were also measured for each timepoint. Briefly, for the TP analysis, the method of Singleton and Rossi with modification (Singleton and Rossi, 1965): Folin-Ciocaletau reagent (1:10) was prepared and 100µL of this was added to 20µL of sample or standard in a 96-well plate, followed by 70µL distilled water. After 5 minutes an additional 70µL sodium carbonate solution (1.1M) was added. After a 2 hour incubation step absorbance was measured at 765nm against gallic acid calibrators (0-500µg/mL in methanol). For the FRAP measurement, the method of Benzie and Stain (Benzie and Strain, 1996) was followed with the following modifications: 225 µL FRAP Reagent (2mM ferric chloride hexahydrate, 1mM 2,4,6-tripyridyl-s-triazine in acetate buffer, pH 3.6) were added to 25µL sample or standards. Absorbance was measured at 593nm after four-minute incubation against Fe^{2+} (ferrous sulphate heptahydrate) calibrators (0 - 1.0mM). For the

measurement of urinary AOPPs the method of Witko et al. was used (WitkoSarsat et al., 1996) with modification: urine samples were diluted 1:5 in PBS. In a 96-well plate 10µL potassium iodide (1.16M) were added to 200µL of sample (diluted 1:5) or standard followed by 20µL acetic acid. Absorbance was measured immediately at 340nm against chloramine-T standards (0-100µM). All measurements were performed in duplicate and the coefficient of variation (CV%) for every assay was <10%.

Statistical analysis

Data were checked for normality and homoscedasticity using the Kolmogorov-Smirnoff test, and for skewness. The Student's t-test, one way-ANOVA and χ^2 (or their non-parametric equivalents) were used to examine the differences among groups. Relationships between continuous variables were tested using Pearson's/Spearman's correlation. Tukey's post-hoc test was used to adjust for multiple comparisons. Changes in dietary intake over the study were compared between the two groups using Repeated Measures ANOVA. All analyses were performed using PASW Statistics 19.0.0 (IBM, SPSS Software, Armonk, NY, USA) and statistical significance was taken as p<0.05.

6.3 Results

Women in the intervention and control groups were similar in terms of age, height, weight, BMI and WC. No significant differences were observed between ethnicity, parity, smoking habits, relationship status and living arrangements between the two groups.

Table 6.1 Fruit, vegetable and polyphenol intake of participants before and after the intervention

		Control (n=55)	Intervention (n=53)	p-value
Whole vegetable intake (80g portions/d)	*Before*	0.70±0.7	0.83±0.9	
	After	1.35±1.6	0.91±0.8	
	Control vs. Intervention (RMANOVA)			*0.41*
Whole fruit intake (80g portions/d)	*Before*	1.41±1.1	1.81±1.8	
	After	1.61±1.6	1.37±1.5	
	Control vs. Intervention (RMANOVA)			*0.53*
Total F&V intake (80g portions/d)	*Before*	4.15±1.8	5.16±2.6	
	After	5.06±3.1	4.94±3.6	
	Control vs. Intervention (RMANOVA)			*0.05*
Total F&V intake excl juice,jam,smoothie (80g portions/d)	*Before*	2.83±1.6	3.16±2.1	
	After	3.52±2.7	3.03±1.9	
	Control vs. Intervention (RMANOVA)			0.97
Total polyphenol intake (mg/d)	*Before*	449.45±419.4	431.16±368.4	
	After	465.03±567.1	486.71±476.9	
	Control vs. Intervention (RMANOVA)			0.98

At baseline, whole fruit intake ranged from 0 to 8.7portions/d and whole vegetable intake ranged from 0 to 3.7portions/d. The total intake of fruit and vegetables (fruit and vegetables included) ranged from 0.5 to 15.7portions/d with substantial contribution from jams, juices and smoothies (consumption range 0-10.2portions/d). At baseline, the two groups had similar whole fruit intake (portions/d: 1.5±1.2 vs. 1.7±1.6, control vs. intervention; p=0.29) and whole vegetable intake (portions/d: 1.2±1.5 vs. 0.9±0.8, control vs. intervention; p=0.07). At baseline, the total fruit and vegetable intake of the intervention group was significantly higher than the control (p=0.01) but only when fruit composites from juices, jams and smoothies were included. Without fruit composites, the remaining fruit and vegetable intake was reduced by more than one and a half portions in both groups, no difference between intervention and control (p=0.29) (Table 6.1). The total polyphenol intakes of individuals, ranged between 1.71mg/day and 2175.14mg/day at baseline with a median intake of 306.58mg/day.

Repeated measures ANOVA analysis showed that there were no significant differences in the dietary changes during the 8-week intervention period between the intervention and control group, despite within-group changes (Table 6.1).

With no evidence that the intervention altered the dietary habits in terms of fruit and vegetable and polyphenol intake, for the remainder of the analysis the control and intervention groups were merged and studied cross-sectionally. From this point onwards, instead of referring to control and intervention groups, the results will be presented as associations at 16-18[+6] weeks and 27-28[+6] weeks gestation.

Association between lifestyle/dietary parameters and glycation-related markers

Fructosamine and urinary AOPPs levels fell significantly by about 3% and 22%, respectively, between 16-18^{+6} weeks and 27-28^{+6} weeks gestation (p<0.001 and p=0.03, respectively). A similar reduction was shown for urinary FRAP levels, while sRAGE and TP levels remained unchanged (Table 6.2).

Table 6.2 Levels of glycation and antioxidant status markers at 16-18^{+6} and 27-28^{+6} weeks gestation

	Week Gestation		
	16-18^{+6}	27-28^{+6}	p-value
	Mean±SD	Mean±SD	
Fructosamine (µM)	195.79±17.9	189.51±18.9	<0.001
AOPPs (µM)	18.27±12.0	14.17±8.1	0.03
Total Phenol (µg/mL gallic acid equiv)	236.88±80.7	209.62±72.8	0.07
FRAP (mM Fe2+ equiv.)	1.26±0.7	1.17±0.6	0.02
sRAGE (pg/mL)	203.69±110.7	191.02±104.7	0.05

Women with higher BMI at baseline had lower glycation, measured as fructosamine, at 16-18^{+6} gestation (p=0.03). No difference in glycation levels were seen, though, between different BMI groups. At 27-28^{+6} weeks gestation there was no relationship between BMI and glycation levels. No relationship was found between BMI and AOPPs or sRAGE levels.

Smoking (n=7) was inversely correlated with fructosamine values at 16-18^{+6} and 27-28^{+6} weeks gestation (p=0.02 and p=0.003, respectively). Current smokers had lower fructosamine levels than those who never smoked, especially at 27-28^{+6} weeks gestation (p=0.051 and p=0.004 at 16-18^{+6} and 27-28^{+6} weeks respectively) (Figure 6.1). Women who were smoking during pregnancy also had significantly higher urinary AOPPs at 27-28^{+6} weeks gestation than never smokers (p=0.02). No difference among the smoking groups were seen at 16-18^{+6} weeks gestation (p=0.25) (Figure 6.2). No association was found between smoking and sRAGE values (p=0.57, 16-18^{+6} wks; p=0.53, 27-28^{+6} wks).

At 16-18^{+6} weeks gestation, TP values were positively correlated with AOPPs values (r=0.57, p<0.001) and so were FRAP values (r=0.22, p=0.02). Levels of fructosamine and sRAGE were not correlated with any other variables. Similar findings were documented at 27-28^{+6} weeks gestation with positive correlations between TP, FRAP and AOPPs levels (r=0.67, p<0.001 and r=0.23, p=0.02 for TP and FRAP respectively). At this timepoint, there was no detectable correlation between urinary TP levels and sRAGE using the p-value cut-off of 0.05, (r=-0.19, p=0.056). However, when women were split in quartiles of TP values, those in the highest quartile of urinary TP, had

significantly higher urinary AOPPs (p<0.001) and lower sRAGE concentrations, at 16-18[+6] weeks gestation (p=0.04) (Table3). The same was documented for AOPPs at 27-28[+6] weeks gestation (TP Q1 vs Q4, p<0.001) but the relationship between TP and sRAGE levels was no longer significant (p=0.09). No differences were seen among women in the higher quartile of urinary FRAP values and those in the lowest quartile in terms of AOPPs, fructosamine and sRAGE levels at any timepoint.

Figure 6.13 Fructosamine levels according to smoking status at 16-18[+6] and 27-28[+6] weeks gestation
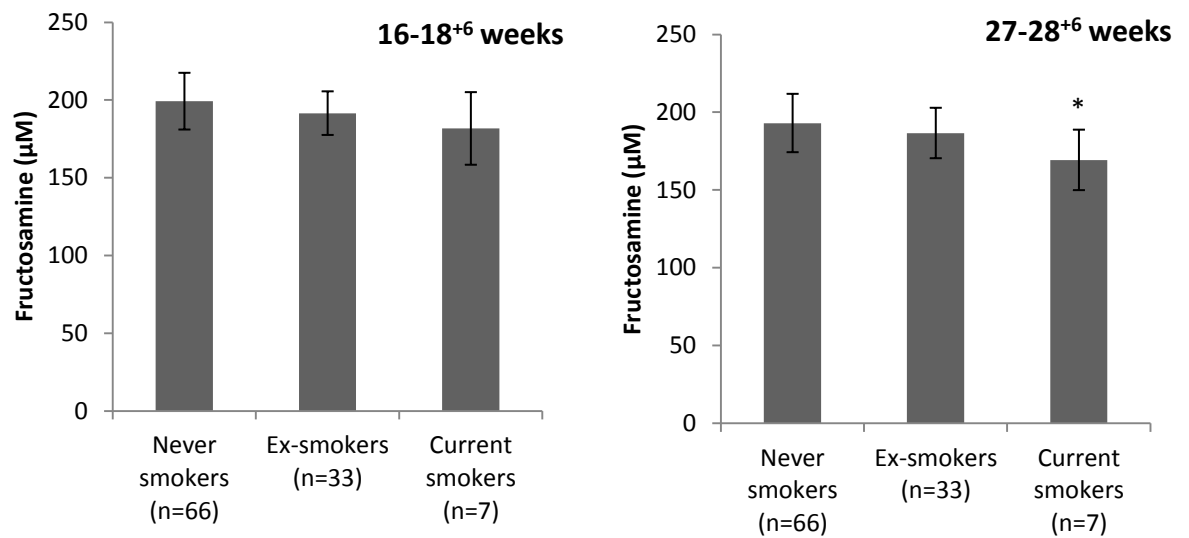
Figure 6.14 AOPPs levels according to smoking status at 16-18[+6] and 27-28[+6] weeks gestation
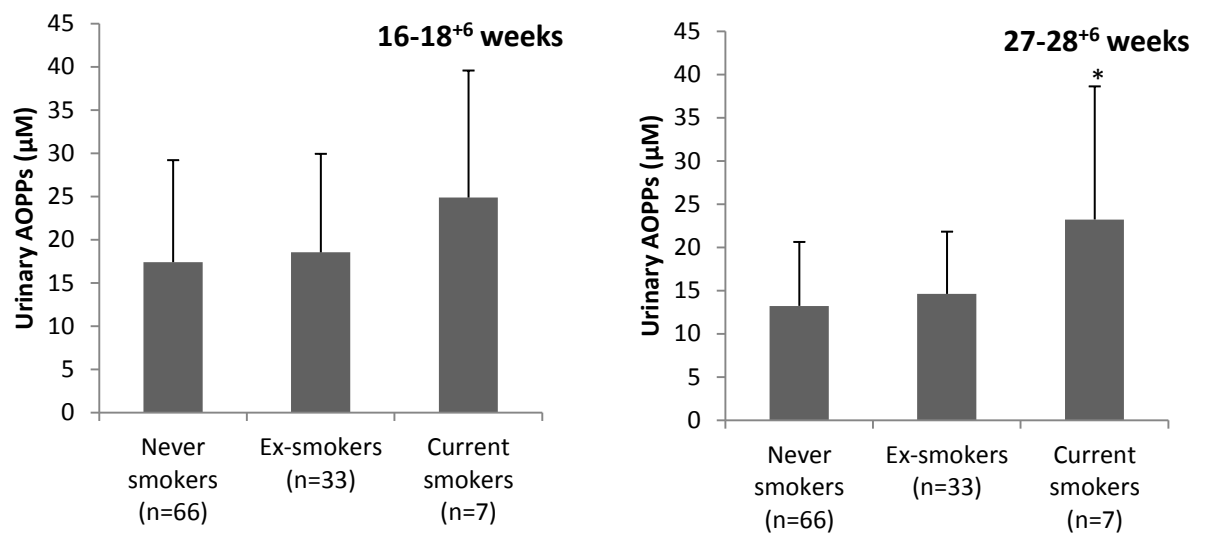
Among the dietary variables, there was no significant interaction between fruit and vegetable intake and fructosamine levels at any time point. That was also the case for urinary AOPPs values. Consuming more than 5 portions of fruit and vegetable per day did not have an impact on fructosamine, AOPPs, sRAGE and FRAP values at either timepoint.

On the other hand, total polyphenol intake was negatively correlated with fructosamine values at 16-18^{+6} and 27-28^{+6} weeks gestation (r=-0.21, p=0.003 and r=-0.27, p=0.008 respectively), although no association was found between total polyphenol intake and urinary AOPPs. A negative correlation between total polyphenol intake and sRAGE levels was observed at 27-28^{+6} weeks gestation (r=-0.26, p=0.01) but not at 16-18^{+6} weeks gestation (r=0.03, p=0.79). When split in quartiles according to dietary polyphenol intake, those with the highest intake (Q4), had significantly lower fructosamine and sRAGE levels (p=0.01 and p=0.03, respectively) at 27-28^{+6} weeks gestation than those with the lowest polyphenol intake (Q1) but not at 16-18^{+6} weeks gestation (Figure 6.3).

Figure 6.15 Fructosamine and sRAGE levels according to quartile total polyphenol intake at 27-28^{+6} weeks gestation
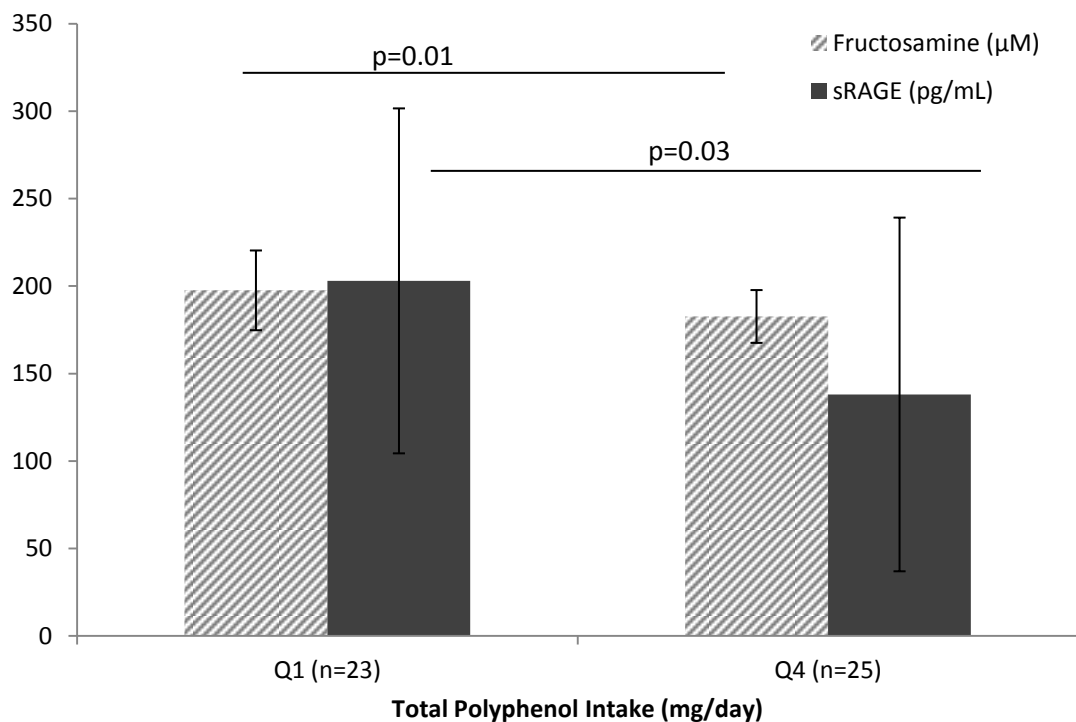
Associations between maternal diet, biomarkers and pregnancy complications

Prevalences of pregnancy complications are shown in Table 6.3. The development of preeclampsia was not associated with maternal AOPPs, fructosamine, sRAGE, FRAP and TP values neither at 16-18^{+6} weeks nor at 27-28^{+6} weeks gestation. Maternal dietary habits, in terms of fruit and vegetable intake or total polyphenol intake were also not associated with the development of preeclampsia. On the other hand, women who developed severe preeclampsia had higher AOPPs at 27-28^{+6} weeks gestation (p=0.01). No association was found between severe preeclampsia and the dietary habits investigated.

Table 6.33 Prevalence of pregnancy complications and adverse neonatal anthropometry

Pregnancy complications	Without n (%)	With n (%)
Preeclampsia	96 (92.3)	8(7.7)
Severe Preeclampsia	99 (95.2)	5 (4.8)
Gestational Diabetes	79 (85.9)	13 (14.1)
Neonatal Anthropometry		
Macrosomia	90 (89.1)	11 (12.9)
Small-for-Gestational Age	88 (87.1)	13(12.9)

Women diagnosed with gestational diabetes were more likely to have elevated fructosamine values at 27-28^{+6} weeks gestation (p=0.01). Being diagnosed with gestational diabetes was also associated with lower total fruit and vegetable consumption at 16-18^{+6} weeks gestation (p=0.05) and the same trend was seen for 27-28^{+6} weeks gestation (p=0.052). Women who were diagnosed with gestational diabetes were also more likely to have lower total polyphenol intake at 27-28^{+6} weeks gestation (614.8±712.6 vs 315.3±321.7 mg/d, p=0.048)

Associations between maternal diet, biomarkers and neonatal outcomes

Prevalences of neonatal macrosomia and being born small for gestational age are reported in Table 6.3. No association was found between maternal dietary habits or maternal AOPPs, fructosamine, sRAGE, FRAP and TP values and the likelihood of giving birth to a macrosomic or a small for gestational age infant. A positive correlation between total polyphenol intake at 27-28^{+6} weeks gestation and neonatal birth weight was documented (r=0.18, p=0.03), but this association did not reach significance when women were split in quartiles of polyphenol intake (p=0.11). Maternal AOPPs at 27-28^{+6} weeks gestation were negatively correlated with neonatal head circumference (r=-0.27, p=0.01) while a similar association with neonatal birth weight (r=-0.19) did not reach significance (p=0.06).

6.4 Discussion

This is, to our best knowledge, the first study to focus on the interactions of lifestyle/dietary habits with glycation markers in obese pregnant women and their effect on maternal and neonatal health. Higher total polyphenol intake was negatively associated with fructosamine values during pregnancy. On the other hand, higher urinary TP values and smoking were associated with higher levels of AOPPs. No study, to-date, has focused on the role of diet on sRAGE levels. We showed for the first time that total polyphenol intake and urinary TP are inversely related with sRAGE values during pregnancy. In terms of pregnancy complications, diet only had an impact on the development of GDM, as women with lower total fruit and vegetable intake and lower total polyphenol intake were more likely to be diagnosed with GDM. From the parameters tested none had a significant relationship with pre-eclampsia diagnosis, but increased levels of AOPPs during the 3rd trimester (27-28^{+6} weeks gestation) were found in women diagnosed with severe pre-eclampsia. Glycation markers have previously been associated with neonatal anthropometry (Kurishita et al., 1994) and in the current analysis, we found a positive relationship between maternal total polyphenol intake and neonatal birth weight. The opposite was documented for AOPPs, which were negatively correlated with neonatal birth weight and head circumference. The results of this analysis are the first to indicate a relationship between maternal AOPPs and neonatal anthropometry. Previous reports suggest that prematurity is associated with higher AOPPs in the neonate which correlate with maternal AOPPs levels (Argüelles et al., 2006, Buonocore et al., 2000). In that respect our results agree with the literature that elevated AOPPs may be a risk factor for fetal development.

The associations between dietary variables and/or the biomarkers of interest were not consistent throughout gestation. In fact, levels of protein glycoxidation themselves fluctuated during gestation, with AOPPs and fructosamine significantly decreasing from 16-18^{+6} to 27-28^{+6} weeks gestation and a similar yet not significant trend observed for sRAGE levels. These results are in agreement with previous reports showing a decrease in fructosamine and AOPPs values during the course of pregnancy, with the exception of women who developed gestational diabetes (Li and Yang, 2006, Fialova et al., 2006, Agarwal et al., 2011, Hiramatsu et al., 2012, Germanova et al., 2010). The fluctuations in biomarkers levels during gestation are likely to be related to the physiological changes of pregnancy. Due to these changes an analysis where data from both timepoints would be pulled together to increase the statistical power would not be appropriate.

The negative relationships between BMI and smoking with glycation marker fructosamine were surprising, as previous data in non-pregnant controls showed opposite relationships (Vlassopoulos et al., 2013a, Clair et al., 2011). However, they are in fact in agreement with earlier reports in pregnant women, also showing inverse impacts of smoking and BMI on fructosamine levels (Hiramatsu et al., 2012). Obesity during pregnancy is a risk factors for proteinuria (Macdonald-Wallis et al., 2011, Gribble et al., 1995, Voigt et al., 2011), which could reduce fructosamine by reducing albumin half-life in circulation; unfortunately no data were available at the time of this analysis to investigate this hypothesis. Literature to-date suggests that smoking is associated with lower proteinuria during pregnancy, which is contradictory to our findings (Macdonald-Wallis et al., 2011, Gribble et al., 1995, Voigt et al., 2011). In this small cohort of obese pregnant women, smoking during pregnancy was uncommon, only 7% of the subjects, and it was associated with significantly higher BMI than non-smokers (p=0.002, data not shown). As the sample size of the current study was not large enough for regression analysis to be performed, it is possible that smoking, here, is a co-variate for higher BMI, rather than a risk factor on its own right. Glycation is directly dependent on protein half-life (Austin et al., 1987, Lapolla et al., 2005) and hence lower fructosamine values in smokers and those with a BMI>40 could merely be an indication of increased protein loss instead of a true reduction in glycation. Expressing fructosamine values as a ratio of fructosamine µmole per mg plasma albumin should be considered as a measure to account for changes in plasma protein concentrations.

Looking at AOPPs concentrations, it becomes evident that smoking is associated with significantly higher levels of protein damage, especially during the third trimester. AOPPs are a marker of protein oxidation and potentially glycoxidation, as they include several chromophores like carbonyls and pentosidine (Capeillere-Blandin et al., 2004, Kalousová et al., 2005). AOPPs have similarities with AGEs as they share formation pathways through oxidative stress but are not solely a marker of glycation (Kalousová et al., 2005), which could explain the difference in results obtain from AOPPs measurement and fructosamine measurements. As AOPPs could be derived by a variety of oxidative processes, they can exhibit high variability among individuals (Codoñer-Franch et al., 2012, Matteucci et al., 2001, Qing et al., 2012), which is the case in our analysis (Figures 1 - 2).

Greater dietary polyphenol intake was associated with lower levels of glycation, measured as fructosamine levels, in our analysis, but the same was not seen for measures of fruit and vegetable intake. Even though fruit and vegetables are the main source of polyphenols in the diet, other food products such as spices, tea, coffee and infusions can be significant sources of polyphenol in populations with relatively low fruit and vegetable intakes. In the current analysis, the correlation between total polyphenol intake and fruit and vegetable consumption was poor,

which indicates that other dietary components are the key polyphenol sources. Previous reports have shown a significant reduction in HbA1c levels in prediabetic individuals after polyphenol supplementation (Fukino et al., 2008, Cho et al., 2012) but the evidence is far from being conclusive as studies in healthy volunteers do not support this finding (Evans M., 2012, Miyazaki et al., 2013).

When studying the impact of polyphenols in health, researchers are faced with two common problems: 1) obtaining accurate and representative data of dietary intake and 2) identifying suitable biomarkers of polyphenol intake (Spencer et al., 2008, Ovaskainen et al., 2008, Perez-Jimenez et al., 2010, Mennen et al., 2006). The estimation of the dietary intake of any nutrient based on recall data is dependent on the quality of information provided by the participant, the quality of information available for the food composition and evidently the tools used to obtain and analyse food records. In dietary research, 7-day food records are still considered the gold standard methodology to obtain intake data (Tucker, 2007). This practise has been criticised as being strenuous for the participants as well as time-consuming for researcher to analyse the data (Spencer et al., 2008, Tucker, 2007). Instead of 7-day records, multi-pass dietary recalls have been utilised extensively in research. Although recalls are more practical for both researcher and participants, they are dependent on the participants' memory and may also not be representative of the overall dietary habits (Basiotis et al., 1987, Buzzard et al., 1996, Thompson F. E., 2008). Under-reporting of the dietary intake is a common source of error in dietary recalls and obese participants are more likely to under-report than normal weight participants (Poppitt et al., 1998, Heitmann and Lissner, 1995, Lichtman et al., 1992, Braam et al., 1998). The Goldberg cut-off has been used in order to assess under-reporting (Goldberg et al., 1991). In the current analysis since all the population is of a BMI>30, it was assumed that the degree of under-reporting would be comparable between participants and hence it was not included in the confounding factors.

The second source of error in dietary recalls is introduced in the analysis of the data obtained. In the case of polyphenols, dietary intakes are based on food composition data, which are most often than not a poor representation of the actual values as they fail to incorporate details like seasonality, plant variety, method of preparation that have an important impact on polyphenol content (Spencer et al., 2008, Hammerstone et al., 2000, Spanos et al., 1990). The use of food composition data like PhenolExplorer also present with difficulties as the databases do not always include the totality of items consumed by the participants. In the case of this analysis, the completeness of the databases was sufficient as only five food items (exotic fruits) were not included in the databases and these items were only consumed once and in limited quantities by selected participants.

The use of plasma or urinary biomarkers for polyphenol intake has been proposed as a way to overcome the problems of dietary estimates. TP has been established as a fast and convenient way to measure polyphenol content in urine and plasma, showing acceptable correlation with dietary intake (Medina-Remon et al., 2009, Medina-Remon et al., 2012). Nonetheless, it is a non-specific assay with interference being introduced by sugars, aromatic amines, organic acids, iron, protein and other substances like AGEs present in the urine (Roura et al., 2006). Urinary levels of individual (poly)phenols have been proposed as an alternative approach but there is not sufficient data at the moment to securely suggest which metabolites would be suitable for the assessment of polyphenol intake in epidemiological settings (Spencer et al., 2008, Nielsen et al., 2002, de Vries et al., 1998).

The aforementioned possible errors and biases could provide explanation between the different results obtained in the present studies among variables like total polyphenol intake, fruit and vegetable intake and TP/FRAP levels. Another potential explanation could be the small sample size. Previous epidemiological studies have managed to detect relationships between fruit and vegetable intake and glycation markers but they employed larger samples (Vlassopoulos et al., 2013a). Also in the current analysis the two groups (control and intervention) had a significantly different fruit and vegetable intake at randomisation. This difference was no longer present at the end of the intervention as the control group increased its intake and the intervention group's intake remained unchanged. This could be either a directly attributed to the intervention itself (the increased in fruit and vegetable intake seen in the control group is from higher jam, juice and smoothie food groups restricted for the intervention group) or it could be merely a regression towards the mean. In the case of the latter, the difference observed in baseline is merely out of chance and that would mean that there was no true change in any of the groups during the 8-week intervention period. The same analysis in the full trial will have the potential to detect changes with a smaller effect size and will also allow for the data to be corrected for confounding factors.

6.5 Conclusion

This unique study has highlighted that polyphenol consumption may be negatively associated with glycation. The level of glycation was 8% greater in the lowest quartile of total polyphenol intake compared to the top quartile. Overall total polyphenol intake explained only 5% of variance in glycation. It also provided with preliminary data to link maternal glycoxidation with pregnancy complications and a link between maternal polyphenol consumption and glycoxidation with neonatal anthropometry.

Chapter 7: General discussion and implications for future research

In the past 60 years, the median age of the entire world population has increased by 5 years, and over 25% of the world population is now older than 45 years (WHO, 2014). Ageing and chronic diseases are the main medical concerns in the developed world (Sweet, 2011, WHO, 2010, WHO, 2011). The identification of early signs of disease pathogenesis is vital for prevention and targeting populations at risk in order to reduce morbidity and mortality. Glycation is well-established as an index of control, or otherwise, and a predictor of end-organ damage, for people with type 2 diabetes, an d has been already identified as a marker of chronic disease pathogenesis even in the absence of diabetes (Selvin et al., 2005, Selvin et al., 2014, Selvin et al., 2010, Adams et al., 2009, van't Riet et al., 2012). The process of glycation is therefore relevant to health and disease throughout the lifecycle, with the potential to modify health trajectories (Nedic et al., 2013, Smith et al., 1995, Wolff et al., 1991).

At the beginning of the work for this thesis (2010), evidence was beginning to be raised to suggest that since glycation levels vary considerably, in normoglycaemic, non-diabetic individuals, glycation cannot be solely be related to glucose levels (Selvin et al., 2010). The impact of oxidative stress in the production of AGEs and intermediate glycation products had been already established (Ahmed et al., 2005, Duran-Jimenez et al., 2009, Thornalley et al., 1984, Thornalley, 2003b) and early reports allowed for speculations about a relationship between early glycation and oxidative stress (Selvaraj et al., 2008, Jain, 1998, Jain and Palmer, 1997). At the same time, mechanistic studies were supporting the epidemiological evidence by suggesting that antioxidant vitamins and (poly)phenols have anti-glycative properties (Jain and Palmer, 1997, Kazeem et al., 2012, Verzelloni et al., 2011a, Xie and Chen, 2013, Xi et al., 2008).

This thesis has employed a systematic methodology incorporating skills across the full breadth of Human Nutrition (basic laboratory sciences, clinical trials and epidemiology) with the aim to establish the relationship between early glycation and oxidative stress in normoglycaemia in an iterative scientific progression:

1. Existing epidemiological data were used to identify relationships between proxies for redox status and early glycation in non-diabetic individuals.

2. The associations from epidemiology were explored in mechanistic laboratory studies with high physiological relevance (using physiological concentrations and conditions) to better characterise the associations between oxidative stress/ antioxidants and early glycation.

3. Clinical trial data and biological samples were analysed from a randomised controlled dietary advice trial in obese pregnant women, a group at risk from higher glycation and oxidative stress.   This study provided a sample cohort advised to follow dietary and lifestyle habits with the potential to modulate multiple glycation markers and maternal and neonatal health.

7.1 Research questions and answers

This section summarises the Research Questions posed following the literature review (Chapter 1), and the answers provided by the current thesis.

RQ1: Is redox status associated with glycation in normoglycaemic individuals?

The current thesis showed in chapter 3 that redox status has an important impact on protein glycation in absence of diabetes. Habits like smoking, associated with increased oxidative stress, were shown to be positively associated with HbA1c levels, in a large epidemiological study. These findings were independent of other factors like age, sex, BMI and WC and were consistent with previous reports (Clair et al., 2011). The new data also suggested that vegetable intake, a habit associated with improved redox status, had the opposite (negative) correlation with HbA1c levels, although the relatively narrow range of vegetable consumptions had a weaker correlation.

Currently, some 10 million adults are smoking in the UK. Despite a reduction in the prevalence of smoking in the last 40 years, 200,000 young people aged 11-15 years commence smoking annually. Over the same period, consumptions of fruit a vegetables have fallen, and this trend continues: a 4-6% reduction in the purchase of fresh and processed fruit and vegetable was

reported between 2008 and 2013, with only a quarter of the population meeting the '5-a-DAY' target (five portions of 80gr). This adverse combination of lifestyle habits has marked socio-economic impact, contributing to health inequalities. Individuals in lower income groups are twice as likely to be smokers, and they consume 50% less fruit and vegetable than the more affluent (HSCIC, 2013, AOS, 2013). The clustering of smoking and poor diet quality is not a novel finding but it is still a major source of health inequalities and by promoting glycation it would accelerate biological ageing in this population.

The measurement of HbA1c has been established as the gold-standard method for glucose monitoring, based on the assumption that it is mainly a reflection of glycaemia, with long-term exposure to glucose determining the circulating concentration. This is crudely true for people with diabetes, although HbA1c still varies despite similar glycaemia as indicated by frequent blood glucose testing. HbA1c is also important clinically as a strong predictor of the multiple clinical complications of diabetes. The finding that oxidative stress can have a potential positive impact on HbA1c levels is thus of substantial importance for clinical practice. The present thesis has demonstrated that smoking is associated with higher HbA1c levels, and smokers are more likely to have HbA1c in the 'pre-diabetic' range, an effect which appears probably independent of glycaemia. The Scottish Health Survey did not include data for blood glucose concentrations, so it was impossible to conclude securely whether this association was completely independent of glycaemia. Nonetheless, we provided evidence for increased HbA1c levels with smoking even in a subgroup of the population with good glycaemic control and low systemic inflammation. This population group is unlikely to be experiencing any dysregulation in glucose handling that could lead to impaired fasting or post-prandial glucose. Therefore these results are suggestive of a glucose independent association between smoking and HbA1c. Such associations imply an important margin of misclassification of smokers as pre-diabetics using HbA1c, as compared to the alternative diagnostic criteria based on OGTT. The issue of misclassification of diabetes using HbA1c has been previously debated among clinicians (Bonora and Tuomilehto, 2011) and a recent meta-analysis highlighted the fact that smokers as a group are more likely to be misclassified as diabetic than non-smokers based on HbA1c levels (Soulimane et al., 2014). The present thesis provides an explanation. Misclassification of diabetes is important. It can affect treatment choices and risk management, leading to further financial implications for the health system, and it also has psychological implications for patients and their families (de Lusignan et al., 2012).

RQ2: Could oxidation promote fructosamine production in the presence of physiological glucose concentrations?

RQ2a: Are oxidised proteins more prone to glycation than the native forms?

Glycation is a complex and multi factorial process. Therefore, it is difficult to obtain mechanistic data from *in-vivo* investigations. Mechanisms of glycation have traditionally been studied using *in-vitro* models with limited physiological relevance. The use of supra-physiologically high glucose levels, low protein concentrations and variable incubation durations are some of the key issue resulting in evidence from *in-vitro* studies being treated cautiously (Bourdon et al., 1999, Chesne et al., 2006, Kim and Kim, 2003, Tarwadi and Agte, 2011). In this thesis we aimed to develop a physiologically relevant setting where the interactions between oxidative stress and glycation could be studied.

We showed that exposure to H_2O_2, at a physiological concentration, was associated with higher early glycation at two and four weeks incubation, independently of glucose levels. More importantly at glucose concentration representative of normoglycaemia (5mM) oxidative stress was a prerequisite for significant amounts of glycation to be detected. These findings are in keeping with the hypothesis generated from epidemiology and further support the speculation that glycation, especially fructosamine, might well be a marker of oxidative stress in normoglycaemia. A novel and important finding of this investigation was that oxidative damage had the capacity to promote glycation even if the two did not take place simultaneously. No previous study so far has proposed a mechanism according to which protein/ amino acid oxidation makes the molecule more prone to glycative damage and it is a field requiring further research. The dogma to-date suggests that glycated proteins are more prone to oxidation but the opposite pathway is poorly characterised.

Oxidative damage is more likely to happen before glycative damage, as glycation is a slow process as opposed to oxidation which can happen rapidly especially intracellularly and perhaps at locations closer to the mitochondria which generate free radicals. Moreover, even though early glycative damage can be repaired enzymatically by the removal of the fructosamine moiety (Delpierre et al., 2002, Delpierre et al., 2000), repair of oxidative damage is based on proteolysis (Costa et al., 2007, Friguet, 2006). These indicate that accumulation of oxidative damage is more likely to accumulate prior to glycation, making glycation a secondary damage especially in normoglycaemia.

RQ3: Can phenolic acids reduce glycation in a physiologically relevant *in-vitro* system?

The same in-vitro setting was utilised to explore the potential of phenolic acids to reduce glycation. The antiglycative properties of (poly)phenols have attracted attention lately but no exact mechanism of action is established. The structures required of (poly)phenols in order to exhibit antiglycative capacities were described in a recent review of the literature and interestingly the same structures are associated with higher antioxidant capacities as well (Xie and Chen, 2013). Prior in-vitro investigations, on the other hand, did not manage to see a link between antioxidant properties and antiglycative properties, which led to the speculation that bonding of phenolic acids on the protein may be offering 'physical protection' against glycation (Verzelloni et al., 2011a).

Both these mechanisms were tested in our experimental setting and we provided with evidence that phenolic acids are more likely to inhibit glycation through protein-phenolic acid interactions rather than as antioxidants. Our results suggest that addition of phenolic acids in the solution during the incubation with glucose did not have an impact on glycation but pre-incubation of albumin with phenolic acids led to significant reduction in early glycation when the protein was exposed to a combination of glucose and H_2O_2. Once again the study was designed to replicate physiology to the highest possible degree. A systematic review of the literature was performed prior to the experiment, which informed the choice of phenolic acids used and their concentrations as well as those of glucose and of H_2O_2.

Apart from the interesting mechanistic data provided from the *in-vitro* experiments, they are also important in terms of methodology for human nutrition research. These studies within the present thesis are proof that mechanistic studies with a high relevance to physiology are not impossible to achieve and they should become the usual expected standard in research. Many *in vitro* studies are conducted using phenolic compounds such as quercetin, which are simply not absorbed by the gastrointestinal tract, or metabolised before they ever reach 'target' tissue. Many studies use completely inappropriate supraphysiological concentrations in order to achieve results. The findings of these studies are often contradicting current thinking and that is an important finding as the use of supra-physiological conditions may lead to the false speculation of the potential mechanisms taking place in physiology. In the present case, the previous studies of glycation had to employ very high glucose concentrations because the complexity of glycation, and involvement of oxidative mechanisms, had not been appreciated.

RQ4: Is polyphenol intake negatively correlated to glycation markers in a population at high risk of oxidative stress?

RQ4a: Is polyphenol intake and/or glycation related to maternal health or pregnancy complications?

RQ4b: Is maternal polyphenol intake and/or maternal glycation related to neonatal health?

Even though mechanistic data are pointing towards a negative association between (poly)phenols and glycation, experimental and epidemiological studies are still inconclusive. The majority of epidemiological studies lack information on polyphenol intake and intervention trials often have small sample sizes. We hypothesised based on our previous findings that populations with increased oxidative stress will be at high risk of glycation. Such populations would be most likely to benefit from a higher polyphenol intake in terms of glycation regulation. Glycation, although a marker of ageing and diabetes, has recently attracted attention as a marker of health trajectories throughout the lifecycle and especially during pregnancy for its potential to regulate maternal and neonatal health. A cohort of obese pregnant women was identified and markers of glycation and glycoxidation were studied for potential associations with polyphenol intake and fruit and vegetable intake. The same markers were also assessed in terms of their involvement in determining maternal health during pregnancy and complications, as well as being associated with neonatal health.

We found that higher polyphenol intake was indeed associated with lower glycation and glycoxidation in the mother. There was also a negative association between polyphenol intake and the development of GDM but not pre-eclampsia. The was a weak (r=0.2) but significant correlation showing that, mothers with higher polyphenol intake gave birth to heavier babies, but the association did not persist when diagnosis of macrosomia was the outcome of interest. Glycation and glycoxidation fluctuated significantly in the course of gestation and maternal glycoxidation was significantly higher in women who developed severe pre-eclampsia. Higher maternal glycoxidation (AOPPs levels) was also related to giving birth to lighter babies with a smaller head circumference.

We showed that monitoring glycoxidation may be an important step towards the improvement of complication diagnosis in pregnancy, but fluctuations during pregnancy (reduction during the 3rd trimester) will have to be taken into consideration. Glycoxidation and maternal polyphenol intake were identified as potential factors of fetal programming especially in terms of anthropometry, but studies in larger cohorts are needed in order to reach a clear conclusion.

Interventional protocols during pregnancy usually rely on improving dietary quality or monitoring the weight gain in order to deliver benefits to the mother and the baby without exposing them to risk. In the case of obese pregnancies, such manipulations may be useful but the main risk factor, which is obesity, is still not addressed. Interventions aiming towards a weight reduction of more than 5%, with maintenance of the loss, are associated with significant health benefits, even diabetes reversal (Penn et al., 2013, Lim et al., 2011, Wing et al., 2011, Blackburn, 1995). Weight loss interventions prior to conception may be more effective in protecting the mother and the baby than intervention modifying the quality of diet alone.

7.2 Considerations for the future

Although the importance of glycation as a marker of disease pathogenesis outside of diabetes is becoming clearer, it is not yet fully understood. More epidemiological studies are required to describe the interactions between oxidative stress and glycation, especially in normoglycaemia. The relatively small effect sizes obtained from the analyses as part of this PhD indicate that future investigations will require large sample sizes to have adequate power and also to allow for successful adjustment for confounders. Studies focusing on glycation have so far only focused on traditional glycation markers and rather neglected the importance of sRAGE and RAGE activation in disease pathogenesis, even though they constitute a major pathway of pathogenesis.

As far as polyphenol and antioxidant trials are concerned, there is still much improvement to be done in terms of study design before conclusions can be reached. If the working hypothesis is that polyphenols will exert health benefits via their antioxidant capacity, at least in susceptible individuals, then markers to document such improvements should be included and results on glycation markers, like HbA1c, should be discussed alongside oxidative stress improvements. The measurement of oxidative stress markers, like F2-isoprostanes, 8-hydroxydeoxyguanosine (8-OH-dG), the Comet assay and measurement of protein carbonyls would allow for sensitive and reproducible measurement of lipid, DNA and protein oxidation, providing a better understanding of the oxidative stress status compared to the commonly used TBARS assay (Freese et al., 1999, Hodgson et al., 2002, Janero, 1990, Frei and Higdon, 2003). Unfortunately, the validity of oxidative stress markers has been a source of disagreement among scientists (Halliwell, 2007, Halliwell, 1994) and no gold-standard method has been identified so far (Dalle-Donne et al., 2006). In that context, it is important to highlight that according to the results of this thesis, HbA1c levels themselves might be a novel marker of oxidative stress. The measurement of HbA1c or fructosamine combined with measurements of blood glucose, ideally both fasting and 2-hour

blood glucose, could be used to identify increased oxidative stress. Future studies should be designed to address this question, as it would valuably inform both research and clinical practice.

Sample size and targeting the correct population are two more aspect of study design to be considered. The regulation of redox status is vital for health, with many endogenous regulatory mechanisms, and chronic diseases are likely to reflect only very small perturbations. Dietary factors are likely to play minor parts in regulation of such an important element of the milieu interieur, although subgroups may be more dependent on external factors. Polyphenol supplementation in a relatively healthy population is likely to have at best subtle effect on health markers and hence studies with large sample sizes greater than a hundred are likely to be needed (Cicero et al., 2008). The majority of the studies to-date fall short of that sample size and are hence likely to be underpowered. As a result, beta-errors are likely, and one should be very careful when concluding that polyphenol supplementation has no effect on glycation. The current literature may just describe a lack of power to detect such an effect (if any). Another consideration could be to target the trials towards populations with increased oxidative stress (i.e. obese, smokers), or to identify population subgroups which might have greater susceptibility to oxidative stress with greater dependence on dietary antioxidants. . Such a population may experience a greater benefit in their redox status from polyphenol (or other) supplementation, with the possibility of a knock-on protective effect on glycation markers. This is only a hypothesis and evidence to support this suggestion is not available.

A very important improvement in future study quality will relate to the information available about the supplement used. So far, reports from supplementation studies rarely report in detail the exact composition of the supplements used. Information on the polyphenol composition, the exact dose of each compound, the matrix used to deliver them and even some prior bioavailability data are needed if the results from different trials are to be compared and robust conclusions drawn.

Glycation markers should be picked carefully and the study duration should be sufficient to detect changes. Studies with duration shorter than the biomarker's half-life are not suitable to represent any changes. Fructosamine measurements for a study with duration less than 14-28 days and HbA1c measurements before a 90-day window are most likely to be unable to detect any changes. The field of proteomics and metabolomics are also of great importance in glycation research, since every protein is ultimately a glycation target analysing changes in patterns of protein damage maybe more effective in drawing conclusion compared to single protein approaches.

Finally, when searching for benefits from a polyphenol supplementation trial, one should keep in mind that physical protein damage is only one pathway of glycation-related pathogenesis. RAGE mediated events are also key in the pathogenesis of diseases and are so far under-studied. Studies on epithelial cells and adipocytes suggest that plant derived polyphenol like silymarin, apigenin, diosmin, epigallocatechin gallate, tyrosol and quercetin have the capacity to reduce glycation related inflammation, especially TNF-alpha levels (Chandler et al., 2010, Kawaguchi et al., 2011, Biesalski, 2007) as well as nitric oxide production as a response to RAGE stimulation (Chandler et al., 2010). Although cell culture experiments can be set-up to study the interaction of polyphenols, glycation and inflammation, this is a more complicated task *in-vivo*. Measurement of sRAGE alongside the measurement of AGEs is not currently a common practice. Studies in pregnant women and patients with juvenile idiopathic arthritis (JIA) have showed that sRAGE levels may be more important that AGE levels to predict disease activity and complications (Yu et al., 2012, Myles et al., 2011). Also the calculation of a sRAGE/AGE ratio is a new concept that attempts to serve as a proxy for AGEs available to bind with RAGE (McNair et al., 2010, Ng et al., 2013). With little being known about sRAGE modulation, it is becoming evident that new glycation related markers need to be used to effectively describe the impact of glycation on health.

7.3 Research questions arising from this thesis

Based on the results of this thesis, the following further research questions appear to be valid, hitherto unanswered and of potential importance to inform practice or policy (among others):

- Is the epidemiological association between oxidative stress and glycation truly independent of glucose levels?
- Which is the exact mechanism t for protein oxidation prior to exposure to glucose to promote glycation? Are oxidised amino acids more susceptible to glycative damage?
- Is the protection offered by phenolic acids against glycation mediated through 'physical protection' of glycation sites? If yes, which phenolic acids most effectively bind on the albumin molecule and is the bonding covalent or not?
- Can nutritional interventions to increase polyphenol intake improve maternal and neonatal health during pregnancy? Are there epigenetic effects?
- Does weight loss prior to conception reduce risk of complications for obese women, and improve neonatal health? Does this reflect redox status in pregnancy?

References

ABDEL-WAHAB, Y. H., O'HARTE, F. P., BARNETT, C. R. & FLATT, P. R. 1997. Characterization of insulin glycation in insulin-secreting cells maintained in tissue culture. *J Endocrinol,* 152**,** 59-67.

ADA 2011. Diagnosis and classification of diabetes mellitus. *Diabetes Care,* 34 Suppl 1**,** S62-9.

ADAMS, R. J., APPLETON, S. L., HILL, C. L., WILSON, D. H., TAYLOR, A. W., CHITTLEBOROUGH, C. R., GILL, T. K. & RUFFIN, R. E. 2009. Independent association of HbA(1c) and incident cardiovascular disease in people without diabetes. *Obesity (Silver Spring),* 17**,** 559-63.

AGARWAL, M. M., DHATT, G. S., OTHMAN, Y. & LJUBISAVLJEVIC, M. R. 2011. Gestational Diabetes: An Evaluation of Serum Fructosamine as a Screening Test in a High-Risk Population. *Gynecologic and Obstetric Investigation,* 71**,** 207-212.

AHMAD, M. S., PISCHETSRIEDER, M. & AHMED, N. 2007. Aged garlic extract and S-allyl cysteine prevent formation of advanced glycation endproducts. *Eur J Pharmacol,* 561**,** 32-8.

AHMED, M. U., THORPE, S. R. & BAYNES, J. W. 1986. Identification of N-Epsilon-Carboxymethyllysine as a Degradation Product of Fructoselysine in Glycated Protein. *Journal of Biological Chemistry,* 261**,** 4889-4894.

AHMED, N., BABAEI-JADIDI, R., HOWELL, S. K., THORNALLEY, P. J. & BEISSWENGER, P. J. 2005. Glycated and Oxidized Protein Degradation Products Are Indicators of Fasting and Postprandial Hyperglycemia in Diabetes. *Diabetes Care,* 28**,** 2465-2471.

AHMED, N. & THORNALLEY, P. J. 2002. Chromatographic assay of glycation adducts in human serum albumin glycated in vitro by derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl-carbamate and intrinsic fluorescence. *Biochem J,* 364**,** 15-24.

AIMUKHAMETOVA, G., UKYBASOVA, T., HAMIDULLINA, Z., ZHUBANYSHEVA, K., HARUN-OR-RASHID, M., YOSHIDA, Y., KASUYA, H. & SAKAMOTO, J. 2012. The impact of maternal obesity on mother and neonatal health: study in a tertiary hospital of Astana, Kazakhstan. *Nagoya J Med Sci,* 74**,** 83-92.

ALBERG, A. 2002. The influence of cigarette smoking on circulating concentrations of antioxidant micronutrients. *Toxicology,* 180**,** 121-37.

ALTER, D. A., WIJEYSUNDERA, H. C., FRANKLIN, B., AUSTIN, P. C., CHONG, A., OH, P. I., TU, J. V. & STUKEL, T. A. 2012. Obesity, lifestyle risk-factors, and health service outcomes among healthy middle-aged adults in Canada. *BMC Health Serv Res,* 12**,** 238.

AMADORI, M. 1931. *Atti R. Acad. Nuz. Lincei Mem. Cl. Scr. Fic. Mat. Nut,* 13**,** 72.

AMERICAN DIABETES, A. 2000. Screening for type 2 diabetes. *Diabetes Care,* 23 Suppl 1**,** S20-3.

AMERICAN DIABETES, A. 2011. Diagnosis and classification of diabetes mellitus. *Diabetes Care,* 34 Suppl 1**,** S62-9.

AMERICAN DIABETES, A., EUROPEAN ASSOCIATION FOR THE STUDY OF, D., INTERNATIONAL FEDERATION OF CLINICAL, C., LABORATORY, M. & INTERNATIONAL DIABETES, F. 2007. Consensus statement on the worldwide standardisation of the HbA1c measurement. *Diabetologia,* 50**,** 2042-3.

ANDERSON, S. S., TSILIBARY, E. C. & CHARONIS, A. S. 1993. Nonenzymatic Glycosylation-Induced Modifications of Intact Bovine Kidney Tubular Basement-Membrane. *Journal of Clinical Investigation,* 92**,** 3045-3052.

ANONYMOUS 1995. The relationship of glycemic exposure (HbA1c) to the risk of development and progression of retinopathy in the diabetes control and complications trial. *Diabetes,* 44**,** 968-83.

AOKI, H., NAGAO, J., UEDA, T., STRONG, J. M., SCHONLAU, F., YU-JING, S., LU, Y. & HORIE, S. 2012. Clinical assessment of a supplement of Pycnogenol(R) and L-arginine in Japanese patients with mild to moderate erectile dysfunction. *Phytother Res,* 26**,** 204-7.

AOS, A. O. S. 2013. Smoking statistics

ARGÜELLES, S., MACHADO, M. J., AYALA, A., MACHADO, A. & HERVÍAS, B. 2006. Correlation between circulating biomarkers of oxidative stress of maternal and umbilical cord blood at birth. *Free Radical Research,* 40**,** 565-570.

AUSTIN, G. E., MULLINS, R. H. & MORIN, L. G. 1987. Non-enzymic glycation of individual plasma proteins in normoglycemic and hyperglycemic patients. *Clin Chem,* 33**,** 2220-4.

BAETEN, J. M., BUKUSI, E. A. & LAMBE, M. 2001. Pregnancy complications and outcomes among overweight and obese nulliparous women. *American Journal of Public Health,* 91**,** 436-440.

BAILEY, A. J., SIMS, T. J., AVERY, N. C. & MILES, C. A. 1993. Chemistry of collagen cross-links: glucose-mediated covalent cross-linking of type-IV collagen in lens capsules. *Biochem J,* 296 (Pt 2)**,** 489-96.

BAKER, J. R., ZYZAK, D. V., THORPE, S. R. & BAYNES, J. W. 1993. Mechanism of fructosamine assay: evidence against role of superoxide as intermediate in nitroblue tetrazolium reduction. *Clin Chem,* 39**,** 2460-5.

BANINI, A. E., BOYD, L. C., ALLEN, J. C., ALLEN, H. G. & SAULS, D. L. 2006. Muscadine grape products intake, diet and blood constituents of non-diabetic and type 2 diabetic subjects. *Nutrition,* 22**,** 1137-45.

BARNABY, O. S., CERNY, R. L., CLARKE, W. & HAGE, D. S. 2011. Quantitative analysis of glycation patterns in human serum albumin using 16O/18O-labeling and MALDI-TOF MS. *Clin Chim Acta,* 412**,** 1606-15.

BARR, E. L., BOYKO, E. J., ZIMMET, P. Z., WOLFE, R., TONKIN, A. M. & SHAW, J. E. 2009. Continuous relationships between non-diabetic hyperglycaemia and both cardiovascular disease and all-cause mortality: the Australian Diabetes, Obesity, and Lifestyle (AusDiab) study. *Diabetologia,* 52**,** 415-24.

BARTH, S. W., KOCH, T. C., WATZL, B., DIETRICH, H., WILL, F. & BUB, A. 2012. Moderate effects of apple juice consumption on obesity-related markers in obese men: impact of diet-gene interaction on body fat content. *Eur J Nutr,* 51**,** 841-50.

BASIOTIS, P. P., WELSH, S. O., CRONIN, F. J., KELSAY, J. L. & MERTZ, W. 1987. Number of days of food intake records required to estimate individual and group nutrient intakes with defined confidence. *J Nutr,* 117**,** 1638-41.

BASTA, G., SCHMIDT, A. M. & DE CATERINA, R. 2004. Advanced glycation end products and vascular inflammation: implications for accelerated atherosclerosis in diabetes. *Cardiovasc Res,* 63**,** 582-92.

BASTEK, J. A., BROWN, A. G., FOREMAN, M. N., MCSHEA, M. A., ANGLIM, L. M., ADAMCZAK, J. E. & ELOVITZ, M. A. 2012. The soluble receptor for advanced glycation end products can prospectively identify patients at greatest risk for preterm birth. *Journal of Maternal-Fetal & Neonatal Medicine,* 25**,** 1762-1768.

BASTYR, E. J., STUART, C. A., BRODOWS, R. G., SCHWARTZ, S., GRAF, C. J., ZAGAR, A., ROBERTSON, K. E. & GRP, I. S. 2000. Therapy focused on lowering postprandial glucose, not fasting glucose, may be superior for lowering HbA(1c). *Diabetes Care,* 23**,** 1236-1241.

BASU, A., NEWMAN, E. D., BRYANT, A. L., LYONS, T. J. & BETTS, N. M. 2013. Pomegranate polyphenols lower lipid peroxidation in adults with type 2 diabetes but have no effects in healthy volunteers: a pilot study. *J Nutr Metab,* 2013**,** 708381.

BATES, C. J., LEAN, M. E., MANSOOR, M. A. & PRENTICE, A. 2004. Nutrient intakes; biochemical and risk indices associated with Type 2 diabetes and glycosylated haemoglobin, in the British National Diet and Nutrition Survey of people aged 65 years and over. *Diabet Med,* 21**,** 677-84.

BAYNES, J. W. 1991. Role of Oxidative Stress in Development of Complications in Diabetes. *Diabetes,* 40**,** 405-412.

BAYNES, J. W. 2002. The Maillard hypothesis on aging: time to focus on DNA. *Ann N Y Acad Sci,* 959**,** 360-7.

BEISSWENGER, P. J., HOWELL, S. K., O'DELL, R. M., WOOD, M. E., TOUCHETTE, A. D. & SZWERGOLD, B. S. 2001. α-Dicarbonyls Increase in the Postprandial Period and Reflect the Degree of Hyperglycemia. *Diabetes Care,* 24**,** 726-732.

BENZIE, I. F. & STRAIN, J. J. 1996. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Anal Biochem,* 239**,** 70-6.

BERROU, J., TOSTIVINT, I., VERRECCHIA, F., BERTHIER, C., BOULANGER, E., MAUVIEL, A., MARTI, H. P., WAUTIER, M. P., WAUTIER, J. L., RONDEAU, E. & HERTIG, A. 2009. Advanced glycation end products regulate extracellular matrix protein and protease expression by human glomerular mesangial cells. *Int J Mol Med,* 23**,** 513-20.

BHAGWAT, S. A., HAYTOWITZ, D.B., HOLDEN, J.M. 2013. USDA database for the flavonoid content of selected foods, Release 3.1. USA: USDA.

BIESALSKI, H. K. 2007. Polyphenols and inflammation: basic interactions. *Curr Opin Clin Nutr Metab Care,* 10**,** 724-8.

BIESALSKI, H. K., GRUNE, T., TINZ, J., ZOLLNER, I. & BLUMBERG, J. B. 2010. Reexamination of a meta-analysis of the effect of antioxidant supplementation on mortality and health in randomized trials. *Nutrients,* 2**,** 929-49.

BIXBY, M., SPIELER, L., MENINI, T. & GUGLIUCCI, A. 2005. Ilex paraguariensis extracts are potent inhibitors of nitrosative stress: a comparative study with green tea and wines using a protein nitration model and mammalian cell cytotoxicity. *Life Sci,* 77**,** 345-58.

BLACKBURN, G. 1995. Effect of Degree of Weight Loss on Health Benefits. *Obesity Research,* 3**,** 211s-216s.

BLAKE, G. J., PRADHAN, A. D., MANSON, J. E., WILLIAMS, G. R., BURING, J., RIDKER, P. M. & GLYNN, R. J. 2004. Hemoglobin A(1c) level and future cardiovascular events among women. *Archives of Internal Medicine,* 164**,** 757-761.

BLOCK, G., DIETRICH, M., NORKUS, E. P., MORROW, J. D., HUDES, M., CAAN, B. & PACKER, L. 2002. Factors Associated with Oxidative Stress in Human Populations. *American Journal of Epidemiology,* 156**,** 274-285.

BOBBINK, I. W., DE BOER, H. C., TEKELENBURG, W. L., BANGA, J. D. & DE GROOT, P. G. 1997. Effect of extracellular matrix glycation on endothelial cell adhesion and spreading: involvement of vitronectin. *Diabetes,* 46**,** 87-93.

BOEING, H., WEISGERBER, U. M., JECKEL, A., ROSE, H. J. & KROKE, A. 2000. Association between glycated hemoglobin and diet and other lifestyle factors in a nondiabetic population: cross-sectional evaluation of data from the Potsdam cohort of the European Prospective Investigation into Cancer and Nutrition Study. *Am J Clin Nutr,* 71**,** 1115-22.

BONDIA-PONS, I., RYAN, L. & MARTINEZ, J. A. 2012. Oxidative stress and inflammation interactions in human obesity. *Journal of Physiology and Biochemistry,* 68**,** 701-711.

BONETTI, T. C., BORGES, E., JR., BRAGA, D. P., IACONELLI, A., JR., KLEINE, J. P. & SILVA, I. D. 2013. Intrafollicular soluble receptor for advanced glycation end products (sRAGE) and embryo quality in assisted reproduction. *Reprod Biomed Online,* 26**,** 62-7.

BONORA, E. & TUOMILEHTO, J. 2011. The Pros and Cons of Diagnosing Diabetes With A1C. *Diabetes Care,* 34**,** S184-S190.

BOOTH, A. A., KHALIFAH, R. G., TODD, P. & HUDSON, B. G. 1997. In vitro kinetic studies of formation of antigenic advanced glycation end products (AGEs) - Novel inhibition of post-Amadori glycation pathways. *Journal of Biological Chemistry,* 272**,** 5430-5437.

BOURDON, E., LOREAU, N. & BLACHE, D. 1999. Glucose and free radicals impair the antioxidant properties of serum albumin. *The FASEB Journal,* 13**,** 233-244.

BOUSOVA, I., MARTIN, J., JAHODAR, L., DUSEK, J., PALICKA, V. & DRSATA, J. 2005. Evaluation of in vitro effects of natural substances of plant origin using a model of protein glycoxidation. *Journal of Pharmaceutical and Biomedical Analysis,* 37**,** 957-962.

BOUTZIOS, G., LIVADAS, S., PIPERI, C., VITORATOS, N., ADAMOPOULOS, C., HASSIAKOS, D., IAVAZZO, C. & DIAMANTI-KANDARAKIS, E. 2013. Polycystic ovary syndrome offspring display increased oxidative stress markers comparable to gestational diabetes offspring. *Fertil Steril,* 99**,** 943-50.

BOYLE, S. P., DOBSON, V. L., DUTHIE, S. J., HINSELWOOD, D. C., KYLE, J. A. M. & COLLINS, A. R. 2000. Bioavailability and efficiency of rutin as an antioxidant: a human supplementation study. *European Journal of Clinical Nutrition,* 54**,** 774-782.

BRAAM, L. A. J. L. M., OCKÉ, M. C., BUENO-DE-MESQUITA, H. B. & SEIDELL, J. C. 1998. Determinants of Obesity-related Underreporting of Energy Intake. *American Journal of Epidemiology,* 147**,** 1081-1086.

BREWER, N., WRIGHT, C. S., TRAVIER, N., CUNNINGHAM, C. W., HORNELL, J., PEARCE, N. & JEFFREYS, M. 2008. A New Zealand linkage study examining the associations between A1C concentration and mortality. *Diabetes Care,* 31**,** 1144-9.

BRIGGS, A. D. M., MIZDRAK, A. & SCARBOROUGH, P. 2013. A statin a day keeps the doctor away: comparative proverb assessment modelling study. *BMJ,* 347.

BRILEY, A. L., BARR, S., BADGER, S., BELL, R., CROKER, H., GODFREY, K. M., HOLMES, B., KINNUNEN, T. I., NELSON, S. M., OTENG-NTIM, E., PATEL, N., ROBSON, S. C., SANDALL, J., SANDERS, T., SATTAR, N., SEED, P. T., WARDLE, J. & POSTON, L. 2014. A complex intervention to improve pregnancy outcome in obese women; the UPBEAT randomised controlled trial. *BMC Pregnancy Childbirth,* 14**,** 74.

BRILEY, A. L., HOLMES, B., KINNUNEN, T. I., CROKER, H., BELL, R., SANDERS, T., WARDLE, J., SANDALL, J., ROBSON, S., SATTAR, N., POSTON, L., NELSON, S. & BARR, S. 2011. Development of a Complex Intervention To Improve Outcome in Obese Pregnancies; the UPBEAT Protocol. *Reproductive Sciences,* 18**,** 143a-143a.

BROMLEY C, B. P., GIVEN L, EDS. The Scottish Health Survey 2008. *Edinburgh:The Scottish Government,2009*.

BROMLEY C, S. K., SHELTON N, EDS. The Scottish Health Survey 2003. *Edinburgh:The Scottish Executive,2005*.

BROWN, A. L., LANE, J., COVERLY, J., STOCKS, J., JACKSON, S., STEPHEN, A., BLUCK, L., COWARD, A. & HENDRICKX, H. 2009. Effects of dietary supplementation with the green tea polyphenol epigallocatechin-3-gallate on insulin resistance and associated metabolic risk factors: randomized controlled trial. *Br J Nutr,* 101**,** 886-94.

BROWNLEE, M., CERAMI, A. & VLASSARA, H. 1988. Advanced Products of Nonenzymatic Glycosylation and the Pathogenesis of Diabetic Vascular-Disease. *Diabetes-Metabolism Reviews,* 4**,** 437-451.

BROWNLEE, M., VLASSARA, H., KOONEY, A., ULRICH, P. & CERAMI, A. 1986. Aminoguanidine prevents diabetes-induced arterial wall protein cross-linking. *Science,* 232**,** 1629-32.

BUONOCORE, G., PERRONE, S., LONGINI, M., TERZUOLI, L. & BRACCI, R. 2000. Total hydroperoxide and advanced oxidation protein products in preterm hypoxic babies. *Pediatr Res,* 47**,** 221-4.

BUZZARD, I. M., FAUCETT, C. L., JEFFERY, R. W., MCBANE, L., MCGOVERN, P., BAXTER, J. S., SHAPIRO, A. C., BLACKBURN, G. L., CHLEBOWSKI, R. T., ELASHOFF, R. M. & WYNDER, E. L. 1996. Monitoring Dietary Change in a Low-Fat Diet Intervention Study: Advantages of Using 24-Hour Dietary Recalls vs Food Records. *Journal of the American Dietetic Association,* 96**,** 574-579.

CAI, W., HE, J. C., ZHU, L., CHEN, X., WALLENSTEIN, S., STRIKER, G. E. & VLASSARA, H. 2007. Reduced oxidant stress and extended lifespan in mice exposed to a low glycotoxin diet: association with increased AGER1 expression. *Am J Pathol,* 170**,** 1893-902.

CAI, W., HE, J. C., ZHU, L., CHEN, X., ZHENG, F., STRIKER, G. E. & VLASSARA, H. 2008. Oral glycotoxins determine the effects of calorie restriction on oxidant stress, age-related diseases, and lifespan. *Am J Pathol,* 173**,** 327-36.

CAMARGO, J. L., STIFFT, J. & GROSS, J. L. 2006. The effect of aspirin and vitamins C and E on HbA1c assays. *Clin Chim Acta,* 372**,** 206-9.

CAPEILLERE-BLANDIN, C., GAUSSON, V., DESCAMPS-LATSCHA, B. & WITKO-SARSAT, V. 2004. Biochemical and spectrophotometric significance of advanced oxidized protein products. *Biochim Biophys Acta,* 1689**,** 91-102.

CATALANO, P. M., PRESLEY, L., MINIUM, J. & HAUGUEL-DE MOUZON, S. 2009. Fetuses of Obese Mothers Develop Insulin Resistance in Utero. *Diabetes Care,* 32**,** 1076-1080.

CELEC, P., HODOSY, J., PALFFY, R., GARDLIK, R., HALCAK, L. & OSTATNIKOVA, D. 2013. The short-term effects of soybean intake on oxidative and carbonyl stress in men and women. *Molecules,* 18**,** 5190-200.

CERAMI, C., FOUNDS, H., NICHOLL, I., MITSUHASHI, T., GIORDANO, D., VANPATTEN, S., LEE, A., ALABED, Y., VLASSARA, H., BUCALA, R. & CERAMI, A. 1997. Tobacco smoke is a source of toxic reactive glycation products. *Proceedings of the National Academy of Sciences of the United States of America,* 94**,** 13915-13920.

CERVATO, G., CARABELLI, M., GERVASIO, S., CITTERA, A., CAZZOLA, R. & CESTARO, B. 2000. ANTIOXBDANT PROPERTIES OF OREGANO (ORIGANUM VULGARE) LEAF EXTRACTS. *Journal of Food Biochemistry,* 24**,** 453-465.

CHANDLER, D., WOLDU, A., RAHMADI, A., SHANMUGAM, K., STEINER, N., WRIGHT, E., BENAVENTE-GARCIA, O., SCHULZ, O., CASTILLO, J. & MUNCH, G. 2010. Effects of plant-derived polyphenols on TNF-alpha and nitric oxide production induced by advanced glycation endproducts. *Mol Nutr Food Res,* 54 Suppl 2**,** S141-50.

CHEN, C.-Y., ABELL, A. M., MOON, Y. S. & KIM, K.-H. 2012. An Advanced Glycation End Product (AGE)-Receptor for AGEs (RAGE) Axis Restores Adipogenic Potential of Senescent Preadipocytes through Modulation of p53 Protein Function. *Journal of Biological Chemistry,* 287**,** 44498-44507.

CHEN, L.-H., ZHU, W.-F., LIANG, L., YANG, X.-Z., WANG, C.-L., ZHU, Y.-R. & FU, J.-F. 2014. Relationship between glycated haemoglobin and subclinical atherosclerosis in obese children and adolescents. *Archives of Disease in Childhood,* 99**,** 39-45.

CHENG, H. H., LAI, M. H., HOU, W. C. & HUANG, C. L. 2004. Antioxidant effects of chromium supplementation with type 2 diabetes mellitus and euglycemic subjects. *J Agric Food Chem,* 52**,** 1385-9.

CHESNE, S., RONDEAU, P., ARMENTA, S. & BOURDON, E. 2006. Effects of oxidative modifications induced by the glycation of bovine serum albumin on its structure and on cultured adipose cells. *Biochimie,* 88**,** 1467-77.

CHETYRKIN, S., MATHIS, M., PEDCHENKO, V., SANCHEZ, O. A., MCDONALD, W. H., HACHEY, D. L., MADU, H., STEC, D., HUDSON, B. & VOZIYAN, P. 2011. Glucose Autoxidation Induces Functional Damage to Proteins via Modification of Critical Arginine Residues. *Biochemistry,* 50**,** 6102-6112.

CHO, Y.-Y., BAEK, N.-I., CHUNG, H.-G., JEONG, T.-S., LEE, K. T., JEON, S.-M., KIM, H.-J., MCGREGOR, R. A. & CHOI, M.-S. 2012. Randomized controlled trial of Sajabalssuk (Artemisia princeps Pampanini) to treat pre-diabetes. *European Journal of Integrative Medicine,* 4**,** e299-e308.

CHOI, S. Y., JUNG, S. H., LEE, H. S., PARK, K. W., YUN, B. S. & LEE, K. W. 2008. Glycation inhibitory activity and the identification of an active compound in Plantago asiatica extract. *Phytotherapy Research,* 22**,** 323-329.

CHOWDHURY, T. A. & LASKER, S. S. 1998. Elevated glycated haemoglobin in non-diabetic patients is associated with an increased mortality in myocardial infarction. *Postgraduate Medical Journal,* 74**,** 480-481.

CHU, S. Y., KIM, S. Y., SCHMID, C. H., DIETZ, P. M., CALLAGHAN, W. M., LAU, J. & CURTIS, K. M. 2007. Maternal obesity and risk of cesarean delivery: a meta-analysis. *Obesity Reviews,* 8**,** 385-394.

CHUGH, S. S., WALLNER, E. I. & KANWAR, Y. S. 2003. Renal development in high-glucose ambience and diabetic embryopathy. *Semin Nephrol,* 23**,** 583-92.

CICERO, A. F. G., NASCETTI, S., LOPEZ-SABATER, M. C., ELOSUA, R., SALONEN, J. T., NYYSSONEN, K., POULSEN, H. E., ZUNFT, H. J. F., KIESEWETTER, H., DE LA TORRE, K., COVAS, M. I., KAIKKONEN, J., MURSU, J., KOENBICK, C., BAUMLER, H., GADDI, A. V. & GRP, E. S. 2008. Changes in LDL fatty acid composition as a response to olive oil treatment are inversely related to lipid oxidative damage: The EUROLIVE study. *Journal of the American College of Nutrition,* 27**,** 314-320.

CLAIR, C., BITTON, A., MEIGS, J. B. & RIGOTTI, N. A. 2011. Relationships of Cotinine and Self-Reported Cigarette Smoking With Hemoglobin A(1c) in the US. *Diabetes Care,* 34**,** 2250-2255.

CODOÑER-FRANCH, P., TAVÁREZ-ALONSO, S., MURRIA-ESTAL, R., TORTAJADA-GIRBÉS, M., SIMÓ-JORDÁ, R. & ALONSO-IGLESIAS, E. 2012. Elevated advanced oxidation protein products

(AOPPs) indicate metabolic risk in severely obese children. *Nutrition, Metabolism and Cardiovascular Diseases,* 22**,** 237-243.

COOKE, C. L., BROCKELSBY, J. C., BAKER, P. N. & DAVIDGE, S. T. 2003. The receptor for advanced glycation end products (RAGE) is elevated in women with preeclampsia. *Hypertens Pregnancy,* 22**,** 173-84.

COOPER, A. J., FOROUHI, N. G., YE, Z., BUIJSSE, B., ARRIOLA, L., BALKAU, B., BARRICARTE, A., BEULENS, J. W., BOEING, H., BUCHNER, F. L., DAHM, C. C., DE LAUZON-GUILLAIN, B., FAGHERAZZI, G., FRANKS, P. W., GONZALEZ, C., GRIONI, S., KAAKS, R., KEY, T. J., MASALA, G., NAVARRO, C., NILSSON, P., OVERVAD, K., PANICO, S., RAMON QUIROS, J., ROLANDSSON, O., ROSWALL, N., SACERDOTE, C., SANCHEZ, M. J., SLIMANI, N., SLUIJS, I., SPIJKERMAN, A. M., TEUCHER, B., TJONNELAND, A., TUMINO, R., SHARP, S. J., LANGENBERG, C., FESKENS, E. J., RIBOLI, E., WAREHAM, N. J. & INTERACT, C. 2012. Fruit and vegetable intake and type 2 diabetes: EPIC-InterAct prospective study and meta-analysis. *Eur J Clin Nutr,* 66**,** 1082-92.

COSTA, V., QUINTANILHA, A. & MORADAS-FERREIRA, P. 2007. Protein oxidation, repair mechanisms and proteolysis in Saccharomyces cerevisiae. *IUBMB Life,* 59**,** 293-8.

COUGHLAN, M. T., THORBURN, D. R., PENFOLD, S. A., LASKOWSKI, A., HARCOURT, B. E., SOURRIS, K. C., TAN, A. L., FUKAMI, K., THALLAS-BONKE, V., NAWROTH, P. P., BROWNLEE, M., BIERHAUS, A., COOPER, M. E. & FORBES, J. M. 2009. RAGE-induced cytosolic ROS promote mitochondrial superoxide generation in diabetes. *J Am Soc Nephrol,* 20**,** 742-52.

CREAGER, M. A., LÜSCHER, T. F., OF, P. W. T. A., COSENTINO, F. & BECKMAN, J. A. 2003. Diabetes and Vascular Disease: Pathophysiology, Clinical Consequences, and Medical Therapy: Part I. *Circulation,* 108**,** 1527-1532.

CROZIER, A., JAGANATH, I. B. & CLIFFORD, M. N. 2009. Dietary phenolics: chemistry, bioavailability and effects on health. *Natural Product Reports,* 26**,** 1001-1043.

DAI, J., JONES, D. P., GOLDBERG, J., ZIEGLER, T. R., BOSTICK, R. M., WILSON, P. W., MANATUNGA, A. K., SHALLENBERGER, L., JONES, L. & VACCARINO, V. 2008. Association between adherence to the Mediterranean diet and oxidative stress. *Am J Clin Nutr,* 88**,** 1364-70.

DAIPONMAK, W., SENAKUN, C. & SIRIAMORNPUN, S. 2014. Antiglycation capacity and antioxidant activities of different pigmented Thai rice. *International Journal of Food Science & Technology***,** n/a-n/a.

DALLAS, C., GERBI, A., ELBEZ, Y., CAILLARD, P., ZAMARIA, N. & CLOAREC, M. 2013. Clinical Study to Assess the Efficacy and Safety of a Citrus Polyphenolic Extract of Red Orange, Grapefruit, and Orange (Sinetrol-XPur) on Weight Management and Metabolic Parameters in Healthy Overweight Individuals. *Phytother Res*.

DALLE-DONNE, I., ROSSI, R., COLOMBO, R., GIUSTARINI, D. & MILZANI, A. 2006. Biomarkers of Oxidative Damage in Human Disease. *Clinical Chemistry,* 52**,** 601-623.

DARNELL, H. L., ARNOLD, B., ZIPURSKY, S. L., PAUL, M., DAVID, B. & JAMES 2000. Protein Glycosylation in the ER and Golgi Complex. W. H. Freeman.

DAVIE, S. J., GOULD, B. J. & YUDKIN, J. S. 1992. Effect of vitamin C on glycosylation of proteins. *Diabetes,* 41**,** 167-73.

DE LA MAZA, M. P., URIBARRI, J., OLIVARES, D., HIRSCH, S., LEIVA, L., BARRERA, G. & BUNOUT, D. 2008. Weight increase is associated with skeletal muscle immunostaining for advanced glycation end products, receptor for advanced glycation end products, and oxidation injury. *Rejuvenation Res,* 11**,** 1041-8.

DE LUSIGNAN, S., SADEK, N., MULNIER, H., TAHIR, A., RUSSELL-JONES, D. & KHUNTI, K. 2012. Miscoding, misclassification and misdiagnosis of diabetes in primary care. *Diabetic Medicine,* 29**,** 181-189.

DE VEGT, F., DEKKER, J. M., RUHE, H. G., STEHOUWER, C. D. A., NIJPELS, G., BOUTER, L. M. & HEINE, R. J. 1999. Hyperglycaemia is associated with all-cause and cardiovascular mortality in the Hoorn population: the Hoorn Study. *Diabetologia,* 42**,** 926-931.

DE VRIES, J. H., HOLLMAN, P. C., MEYBOOM, S., BUYSMAN, M. N., ZOCK, P. L., VAN STAVEREN, W. A. & KATAN, M. B. 1998. Plasma concentrations and urinary excretion of the antioxidant

flavonols quercetin and kaempferol as biomarkers for dietary intake. *The American Journal of Clinical Nutrition,* 68**,** 60-5.

DEGROOT, J. 2004. Advanced glycation endproducts in the development of osteoarthritis. *Annals of the Rheumatic Diseases,* 63**,** 31-31.

DEL RIO, D., COSTA, L. G., LEAN, M. E. & CROZIER, A. 2010. Polyphenols and health: what compounds are involved? *Nutr Metab Cardiovasc Dis,* 20**,** 1-6.

DELPIERRE, G., COLLARD, F., FORTPIED, J. & VAN SCHAFTINGEN, E. 2002. Fructosamine 3-kinase is involved in an intracellular deglycation pathway in human erythrocytes. *Biochemical Journal,* 365**,** 801-808.

DELPIERRE, G., RIDER, M. H., COLLARD, F., STROOBANT, V., VANSTAPEL, F., SANTOS, H. & VAN SCHAFTINGEN, E. 2000. Identification, cloning, and heterologous expression of a mammalian fructosamine-3-kinase. *Diabetes,* 49**,** 1627-34.

DEN ENGELSEN, C., VAN DEN DONK, M., GORTER, K. J., SALOME, P. L. & RUTTEN, G. E. 2012. Advanced glycation end products measured by skin autofluorescence in a population with central obesity. *Dermatoendocrinol,* 4**,** 33-8.

DEO, P., GLENN, J. V., POWELL, L. A., STITT, A. W. & AMES, J. M. 2009. Upregulation of oxidative stress markers in human microvascular endothelial cells by complexes of serum albumin and digestion products of glycated casein. *J Biochem Mol Toxicol,* 23**,** 364-72.

DIAMANTI-KANDARAKIS, E., PIPERI, C., PATSOURIS, E., KORKOLOPOULOU, P., PANIDIS, D., PAWELCZYK, L., PAPAVASSILIOU, A. G. & DULEBA, A. J. 2007. Immunohistochemical localization of advanced glycation end-products (AGEs) and their receptor (RAGE) in polycystic and normal ovaries. *Histochem Cell Biol,* 127**,** 581-9.

DORSEY, P. G. & GREENSPAN, P. 2014. Inhibition of Nonenzymatic Protein Glycation by Pomegranate and Other Fruit Juices. *J Med Food*.

DUNCAN, B. B., SCHMIDT, M. I., PANKOW, J. S., BALLANTYNE, C. M., COUPER, D., VIGO, A., HOOGEVEEN, R., FOLSOM, A. R. & HEISS, G. 2003. Low-Grade Systemic Inflammation and the Development of Type 2 Diabetes: The Atherosclerosis Risk in Communities Study. *Diabetes,* 52**,** 1799-1805.

DURAN-JIMENEZ, B., DOBLER, D., MOFFATT, S., RABBANI, N., STREULI, C. H., THORNALLEY, P. J., TOMLINSON, D. R. & GARDINER, N. J. 2009. Advanced glycation end products in extracellular matrix proteins contribute to the failure of sensory nerve regeneration in diabetes. *Diabetes,* 58**,** 2893-903.

EDWARDS, B. K., WARD, E., KOHLER, B. A., EHEMAN, C., ZAUBER, A. G., ANDERSON, R. N., JEMAL, A., SCHYMURA, M. J., LANSDORP-VOGELAAR, I., SEEFF, L. C., VAN BALLEGOOIJEN, M., GOEDE, S. L. & RIES, L. A. G. 2010. Annual report to the nation on the status of cancer, 1975-2006, featuring colorectal cancer trends and impact of interventions (risk factors, screening, and treatment) to reduce future rates. *Cancer,* 116**,** 544-573.

EL-SALEM, K., AMMARI, F., KHADER, Y. & DHAIMAT, O. 2007. Elevated glycosylated hemoglobin is associated with subclinical neuropathy in neurologically asymptomatic type-2 diabetic patients: A prospective study. *European Journal of Neurology,* 14**,** 251-251.

ESPOSITO, K., DI PALO, C., MAIORINO, M. I., PETRIZZO, M., BELLASTELLA, G., SINISCALCHI, I. & GIUGLIANO, D. 2011. Long-term effect of mediterranean-style diet and calorie restriction on biomarkers of longevity and oxidative stress in overweight men. *Cardiol Res Pract,* 2011**,** 293916.

EVANS M., S. P. A. G. N. 2012. Bioavailability of Citrus Polymethoxylated Flavones and Their Biological Role in Metabolic Syndrome and Hyperlipidemia. *In:* NOREDDIN, D. A. (ed.) *Readings in Advanced Pharmacokinetics - Theory, Methods and Applications.* InTech.

FAGARD, R. H. & NILSSON, P. M. 2009. Smoking and diabetes--the double health hazard! *Prim Care Diabetes,* 3**,** 205-9.

FESTA, A., D'AGOSTINO, R., WILLIAMS, K., KARTER, A. J., MAYER-DAVIS, E. J., TRACY, R. P. & HAFFNER, S. M. 2001. The relation of body fat mass and distribution to markers of chronic inflammation. *International journal of obesity and related metabolic disorders : journal of the International Association for the Study of Obesity,* 25**,** 1407-1415.

FIALOVA, L., MALBOHAN, I., KALOUSOVA, M., SOUKUPOVA, J., KROFTA, L., STIPEK, S. & ZIMA, T. 2006. Oxidative stress and inflammation in pregnancy. *Scand J Clin Lab Invest,* 66**,** 121-7.

FINESCHI, S., DE CUNTO, G., FACCHINETTI, F., CIVELLI, M., IMBIMBO, B. P., CARNINI, C., VILLETTI, G., LUNGHI, B., STOCHINO, S., GIBBONS, D. L., HAYDAY, A., LUNGARELLA, G. & CAVARRA, E. 2013. Receptor for advanced glycation end products contributes to postnatal pulmonary development and adult lung maintenance program in mice. *Am J Respir Cell Mol Biol,* 48**,** 164-71.

FOROUHI, N. G., SATTAR, N. & MCKEIGUE, P. M. 2001. Relation of C-reactive protein to body fat distribution and features of the metabolic syndrome in Europeans and South Asians. *International journal of obesity and related metabolic disorders : journal of the International Association for the Study of Obesity,* 25**,** 1327-1331.

FREESE, R., BASU, S., HIETANEN, E., NAIR, J., NAKACHI, K., BARTSCH, H. & MUTANEN, M. 1999. Green tea extract decreases plasma malondialdehyde concentration but does not affect other indicators of oxidative stress, nitric oxide production, or hemostatic factors during a high-linoleic acid diet in healthy females. *European Journal of Nutrition,* 38**,** 149-157.

FREI, B. & HIGDON, J. V. 2003. Antioxidant activity of tea polyphenols in vivo: Evidence from animal studies. *Journal of Nutrition,* 133**,** 3275s-3284s.

FREI, B., YAMAMOTO, Y., NICLAS, D. & AMES, B. N. 1988. Evaluation of an isoluminol chemiluminescence assay for the detection of hydroperoxides in human blood plasma. *Anal Biochem,* 175**,** 120-30.

FRIGUET, B. 2006. Oxidized protein degradation and repair in ageing and oxidative stress. *FEBS Letters,* 580**,** 2910-2916.

FRÖHLICH, M., SUND, M., LÖWEL, H., IMHOF, A., HOFFMEISTER, A. & KOENIG, W. 2003. Independent association of various smoking characteristics with markers of systemic inflammation in men: Results from a representative sample of the general population (MONICA Augsburg Survey 1994/95). *European Heart Journal,* 24**,** 1365-1372.

FU, M. X., WELLS-KNECHT, K. J., BLACKLEDGE, J. A., LYONS, T. J., THORPE, S. R. & BAYNES, J. W. 1994. Glycation, glycoxidation, and cross-linking of collagen by glucose. Kinetics, mechanisms, and inhibition of late stages of the Maillard reaction. *Diabetes,* 43**,** 676-83.

FUKINO, Y., IKEDA, A., MARUYAMA, K., AOKI, N., OKUBO, T. & ISO, H. 2008. Randomized controlled trial for an effect of green tea-extract powder supplementation on glucose abnormalities. *Eur J Clin Nutr,* 62**,** 953-60.

FUKINO, Y., SHIMBO, M., AOKI, N., OKUBO, T. & ISO, H. 2005. Randomized controlled trial for an effect of green tea consumption on insulin resistance and inflammation markers. *J Nutr Sci Vitaminol (Tokyo),* 51**,** 335-42.

GAENS, K. H., STEHOUWER, C. D. & SCHALKWIJK, C. G. 2013. Advanced glycation endproducts and its receptor for advanced glycation endproducts in obesity. *Curr Opin Lipidol,* 24**,** 4-11.

GAENS, K. H. J., NIESSEN, P. M. G., RENSEN, S. S., BUURMAN, W. A., GREVE, J. W. M., DRIESSEN, A., WOLFS, M. G. M., HOFKER, M. H., BLOEMEN, J. G., DEJONG, C. H., STEHOUWER, C. D. A. & SCHALKWIJK, C. G. 2012. Endogenous formation of Nε-(carboxymethyl)lysine is increased in fatty livers and induces inflammatory markers in an in vitro model of hepatic steatosis. *Journal of hepatology,* 56**,** 647-655.

GALKINA, E. & LEY, K. 2006. Leukocyte recruitment and vascular injury in diabetic nephropathy. *Journal of the American Society of Nephrology,* 17**,** 368-377.

GALTIER-DEREURE, F., BOEGNER, C. & BRINGER, J. 2000. Obesity and pregnancy: complications and cost. *The American Journal of Clinical Nutrition,* 71**,** 1242s-1248s.

GERMANOVA, A., KOUCKY, M., HAJEK, Z., PARIZEK, A., ZIMA, T. & KALOUSOVA, M. 2010. Soluble receptor for advanced glycation end products in physiological and pathological pregnancy. *Clin Biochem,* 43**,** 442-6.

GEROLDI, D., FALCONE, C., EMANUELE, E., D'ANGELO, A., CALCAGNINO, M., BUZZI, M. P., SCIOLI, G. A. & FOGARI, R. 2005. Decreased plasma levels of soluble receptor for advanced glycation end-products in patients with essential hypertension. *J Hypertens,* 23**,** 1725-9.

GKOGKOLOU, P. & BOHM, M. 2012. Advanced glycation end products: Key players in skin aging? *Dermatoendocrinol,* 4**,** 259-70.

GOLDBERG, G. R., BLACK, A. E., JEBB, S. A., COLE, T. J., MURGATROYD, P. R., COWARD, W. A. & PRENTICE, A. M. 1991. Critical evaluation of energy intake data using fundamental principles of energy physiology: 1. Derivation of cut-off limits to identify under-recording. *Eur J Clin Nutr,* 45**,** 569-81.

GOLDBERG, T., CAI, W., PEPPA, M., DARDAINE, V., BALIGA, B. S., URIBARRI, J. & VLASSARA, H. 2004. Advanced glycoxidation end products in commonly consumed foods. *Journal of the American Dietetic Association,* 104**,** 1287-1291.

GOLDSTEIN, D. E., LITTLE, R. R., LORENZ, R. A., MALONE, J. I., NATHAN, D., PETERSON, C. M. & SACKS, D. B. 2004. Tests of glycemia in diabetes. *Diabetes Care,* 27**,** 1761-73.

GOVERNMENT, T. S. 2008. Better Coronary Heart Disease and Stroke Care - a consultation document.

GRIBBLE, R. K., FEE, S. C. & BERG, R. L. 1995. The value of routine urine dipstick screening for protein at each prenatal visit. *American Journal of Obstetrics and Gynecology,* 173**,** 214-217.

GRIMM, T., SKRABALA, R., CHOVANOVA, Z., MUCHOVA, J., SUMEGOVA, K., LIPTAKOVA, A., DURACKOVA, Z. & HOGGER, P. 2006. Single and multiple dose pharmacokinetics of maritime pine bark extract (pycnogenol) after oral administration to healthy volunteers. *BMC Clin Pharmacol,* 6**,** 4.

GRUNDY, S. M., CLEEMAN, J. I., MERZ, C. N., BREWER, H. B., JR., CLARK, L. T., HUNNINGHAKE, D. B., PASTERNAK, R. C., SMITH, S. C., JR. & STONE, N. J. 2004. Implications of recent clinical trials for the National Cholesterol Education Program Adult Treatment Panel III guidelines. *Circulation,* 110**,** 227-39.

GUEDES, S., VITORINO, R., DOMINGUES, R., AMADO, F. & DOMINGUES, P. 2009. Oxidation of bovine serum albumin: identification of oxidation products and structural modifications. *Rapid Commun Mass Spectrom,* 23**,** 2307-15.

GUERIN-DUBOURG, A., CATAN, A., BOURDON, E. & RONDEAU, P. 2012. Structural modifications of human albumin in diabetes. *Diabetes Metab,* 38**,** 171-8.

GUGLIUCCI, A., BASTOS, D. H., SCHULZE, J. & SOUZA, M. F. 2009. Caffeic and chlorogenic acids in Ilex paraguariensis extracts are the main inhibitors of AGE generation by methylglyoxal in model proteins. *Fitoterapia,* 80**,** 339-44.

GUL, A., RAHMAN, M. A., SALIM, A. & SIMJEE, S. U. 2009. Advanced glycation end products in senile diabetic and nondiabetic patients with cataract. *J Diabetes Complications,* 23**,** 343-8.

GUTIERREZ, R. M. P. 2012. Inhibition of Advanced Glycation End-Product Formation by Origanum majorana L. In Vitro and in Streptozotocin-Induced Diabetic Rats. *Evidence-Based Complementary and Alternative Medicine*.

HAEDERSDAL, S., SALVIG, J. D., AABYE, M., THORBALL, C. W., RUHWALD, M., LADELUND, S., EUGEN-OLSEN, J. & SECHER, N. J. 2013. Inflammatory markers in the second trimester prior to clinical onset of preeclampsia, intrauterine growth restriction, and spontaneous preterm birth. *Inflammation,* 36**,** 907-13.

HALLIWELL, B. 1994. Free radicals, antioxidants, and human disease: curiosity, cause, or consequence? *The Lancet,* 344**,** 721-724.

HALLIWELL, B. 2007. Biochemistry of oxidative stress. *Biochem Soc Trans,* 35**,** 1147-50.

HAMMERSTONE, J. F., LAZARUS, S. A. & SCHMITZ, H. H. 2000. Procyanidin content and variation in some commonly consumed foods. *J Nutr,* 130**,** 2086S-92S.

HAN, T. S., SATTAR, N. & LEAN, M. 2006. ABC of obesity. Assessment of obesity and its clinical implications. *BMJ,* 333**,** 695-8.

HAO, L., NOGUCHI, S., KAMADA, Y., SASAKI, A., MATSUDA, M., SHIMIZU, K., HIRAMATSU, Y. & NAKATSUKA, M. 2008. Adverse effects of advanced glycation end products on embryonal development. *Acta Med Okayama,* 62**,** 93-9.

HARDING, A.-H., SARGEANT, L. A., WELCH, A., OAKES, S., LUBEN, R. N., BINGHAM, S., DAY, N. E., KHAW, K.-T. & WAREHAM, N. J. 2001. Fat Consumption and HbA1c Levels: The EPIC-Norfolk Study. *Diabetes Care,* 24**,** 1911-1916.

HARDING, A. H., DAY, N. E., KHAW, K. T., BINGHAM, S. A., LUBEN, R. N., WELSH, A. & WAREHAM, N. J. 2004. Habitual fish consumption and glycated haemoglobin: the EPIC-Norfolk study. *Eur J Clin Nutr,* 58**,** 277-84.

HARDING, J. J. & BESWICK, H. T. 1988. The possible contribution of glucose autoxidation to protein modification of diabetes. *Biochem J,* 249**,** 617-8.

HARRIS, C., CUERRIER, A., LAMONT, E., HADDAD, P., ARNASON, J., BENNETT, S. L. & JOHNS, T. 2014. Investigating Wild Berries as a Dietary Approach to Reducing the Formation of Advanced Glycation Endproducts: Chemical Correlates of In Vitro Antiglycation Activity. *Plant Foods for Human Nutrition***,** 1-7.

HARRIS, C. S., BEAULIEU, L. P., FRASER, M. H., MCINTYRE, K. L., OWEN, P. L., MARTINEAU, L. C., CUERRIER, A., JOHNS, T., HADDAD, P. S., BENNETT, S. A. & ARNASON, J. T. 2011. Inhibition of advanced glycation end product formation by medicinal plant extracts correlates with phenolic metabolites and antioxidant activity. *Planta Med,* 77**,** 196-204.

HARSEM, N. K., BRAEKKE, K., TORJUSSEN, T., HANSSEN, K. & STAFF, A. C. 2008. Advanced glycation end products in pregnancies complicated with diabetes mellitus or preeclampsia. *Hypertens Pregnancy,* 27**,** 374-86.

HASHIM, Y. Z., ENG, M., GILL, C. I., MCGLYNN, H. & ROWLAND, I. R. 2005. Components of olive oil and chemoprevention of colorectal cancer. *Nutr Rev,* 63**,** 374-86.

HEERWAGEN, M. J. R., MILLER, M. R., BARBOUR, L. A. & FRIEDMAN, J. E. 2010. Maternal obesity and fetal metabolic programming: a fertile epigenetic soil. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology,* 299**,** R711-R722.

HEGDE, P., CHANDRAKASAN, G. & CHANDRA, T. 2002. Inhibition of collagen glycation and crosslinking in vitro by methanolic extracts of Finger millet (Eleusine coracana) and Kodo millet (Paspalum scrobiculatum). *Journal of Nutritional Biochemistry,* 13**,** 517.

HEINRICH, J., VALENTOVA, K., VACEK, J., PALIKOVA, I., ZATLOUKALOVA, M., KOSINA, P., ULRICHOVA, J., VRBKOVA, J. & SIMANEK, V. 2013. Metabolic profiling of phenolic acids and oxidative stress markers after consumption of Lonicera caerulea L. fruit. *J Agric Food Chem,* 61**,** 4526-32.

HENNING, S. M., WANG, P., ABGARYAN, N., VICINANZA, R., DE OLIVEIRA, D. M., ZHANG, Y., LEE, R.-P., CARPENTER, C. L., ARONSON, W. J. & HEBER, D. 2013. Phenolic acid concentrations in plasma and urine from men consuming green or black tea and potential chemopreventive properties for colon cancer. *Molecular Nutrition & Food Research,* 57**,** 483-492.

HEITMANN, B. L. & LISSNER, L. 1995. Dietary underreporting by obese individuals--is it specific or non-specific? *Bmj,* 311**,** 986-9.

HERMSDORFF, H. H., PUCHAU, B., VOLP, A. C., BARBOSA, K. B., BRESSAN, J., ZULET, M. A. & MARTINEZ, J. A. 2011. Dietary total antioxidant capacity is inversely related to central adiposity as well as to metabolic and oxidative stress markers in healthy young adults. *Nutr Metab (Lond),* 8**,** 59.

HESLEHURST, N., ELLS, L. J., SIMPSON, H., BATTERHAM, A., WILKINSON, J. & SUMMERBELL, C. D. 2007. Trends in maternal obesity incidence rates, demographic predictors, and health inequalities in 36,821 women over a 15-year period. *Bjog,* 114**,** 187-94.

HESLEHURST, N., RANKIN, J., WILKINSON, J. R. & SUMMERBELL, C. D. 2010. A nationally representative study of maternal obesity in England, UK: trends in incidence and demographic inequalities in 619 323 births, 1989-2007. *Int J Obes (Lond),* 34**,** 420-8.

HESLEHURST, N., SIMPSON, H., ELLS, L. J., RANKIN, J., WILKINSON, J., LANG, R., BROWN, T. J. & SUMMERBELL, C. D. 2008. The impact of maternal BMI status on pregnancy outcomes with immediate short-term obstetric resource implications: a meta-analysis. *Obes Rev,* 9**,** 635-83.

HIRAMATSU, Y., SHIMIZU, I., OMORI, Y. & NAKABAYASHI, M. 2012. Determination of reference intervals of glycated albumin and hemoglobin A1c in healthy pregnant Japanese women and analysis of their time courses and influencing factors during pregnancy. *Endocr J,* 59**,** 145-51.

HO, S.-C., CHANG, P.-W., TONG, H.-T. & YU, P.-Y. 2013. Inhibition of Fluorescent Advanced Glycation End-Products and N-Carboxymethyllysine Formation by Several Floral Herbal Infusions. *International Journal of Food Properties,* 17**,** 617-628.

HODGE, J. E. 1953. Dehydrated Foods - Chemistry of Browning Reactions in Model Systems. *Journal of Agricultural and Food Chemistry,* 1**,** 928-943.

HODGE, J. E. 1955. The Amadori rearrangement. *Adv Carbohydr Chem,* 10**,** 169-205.

HODGSON, J. M., CROFT, K. D., MORI, T. A., BURKE, V., BEILIN, L. J. & PUDDEY, I. B. 2002. Regular ingestion of tea does not inhibit in vivo lipid peroxidation in humans. *Journal of Nutrition,* 132**,** 55-58.

HOFMANN, M. A., DRURY, S., FU, C. F., QU, W., TAGUCHI, A., LU, Y., AVILA, C., KAMBHAM, N., BIERHAUS, A., NAWROTH, P., NEURATH, M. F., SLATTERY, T., BEACH, D., MCCLARY, J., NAGASHIMA, M., MORSER, J., STERN, D. & SCHMIDT, A. M. 1999. RAGE mediates a novel proinflammatory axis: A central cell surface receptor for S100/calgranulin polypeptides. *Cell,* 97**,** 889-901.

HOLT, E. M., STEFFEN, L. M., MORAN, A., BASU, S., STEINBERGER, J., ROSS, J. A., HONG, C.-P. & SINAIKO, A. R. 2009. Fruit and Vegetable Consumption and Its Relation to Markers of Inflammation and Oxidative Stress in Adolescents. *Journal of the American Dietetic Association,* 109**,** 414-421.

HOU, T. H., CHUNG, J. P., CHEN, S. S. & CHANG, T. L. 2013. Antioxidation and antiglycation of 95% ethanolic extracts prepared from the leaves of black nightshade (Solanum nigrum). *Food Science and Biotechnology,* 22**,** 839-844.

HOWARD, G. J. & WEBSTER, T. F. 2013. Contrasting theories of interaction in epidemiology and toxicology. *Environ Health Perspect,* 121**,** 1-6.

HSCIC, H. A. S. C. I. C. 2013. Statistics on Obesity, Physical Activity and Diet: England, 2013.

HU, F. B. 2003. Plant-based foods and prevention of cardiovascular disease: an overview. *The American Journal of Clinical Nutrition,* 78**,** 544S-551S.

HUDA, S. S., BRODIE, L. E. & SATTAR, N. 2010. Obesity in pregnancy: prevalence and metabolic consequences. *Seminars in Fetal and Neonatal Medicine,* 15**,** 70-76.

HULSMANS, M. & HOLVOET, P. 2010. The vicious circle between oxidative stress and inflammation in atherosclerosis. *Journal of Cellular and Molecular Medicine,* 14**,** 70-78.

HUNG, T.-H., LO, L.-M., CHIU, T.-H., LI, M.-J., YEH, Y.-L., CHEN, S.-F. & HSIEH, T. S.-T. A. 2010. A Longitudinal Study of Oxidative Stress and Antioxidant Status in Women With Uncomplicated Pregnancies Throughout Gestation. *Reproductive Sciences,* 17**,** 401-409.

HUNT, J. V. & WOLFF, S. P. 1991. Oxidative Glycation and Free-Radical Production - a Causal Mechanism of Diabetic Complications. *Free Radical Research Communications,* 12-3**,** 115-123.

HUNTER, S. J., BOYD, A. C., O'HARTE, F. P., MCKILLOP, A. M., WIGGAM, M. I., MOONEY, M. H., MCCLUSKEY, J. T., LINDSAY, J. R., ENNIS, C. N., GAMBLE, R., SHERIDAN, B., BARNETT, C. R., MCNULTY, H., BELL, P. M. & FLATT, P. R. 2003. Demonstration of glycated insulin in human diabetic plasma and decreased biological activity assessed by euglycemic-hyperinsulinemic clamp technique in humans. *Diabetes,* 52**,** 492-8.

ILCHMANN, A., BURGDORF, S., SCHEURER, S., WAIBLER, Z., NAGAI, R., WELLNER, A., YAMAMOTO, Y., YAMAMOTO, H., HENLE, T., KURTS, C., KALINKE, U., VIETHS, S. & TODA, M. 2010. Glycation of a food allergen by the Maillard reaction enhances its T-cell immunogenicity: role of macrophage scavenger receptor class A type I and II. *J Allergy Clin Immunol,* 125**,** 175-83 e1-11.

INABA, M., OKUNO, S., KUMEDA, Y., YAMADA, S., IMANISHI, Y., TABATA, T., OKAMURA, M., OKADA, S., YAMAKAWA, T., ISHIMURA, E., NISHIZAWA, Y. & GROUP, A. T. O. C. E. R. 2007. Glycated Albumin Is a Better Glycemic Indicator than Glycated Hemoglobin Values in Hemodialysis Patients with Diabetes: Effect of Anemia and Erythropoietin Injection. *Journal of the American Society of Nephrology,* 18**,** 896-903.

ISIK, B., CEYLAN, A. & ISIK, R. 2007. Oxidative stress in smokers and non-smokers. *Inhal Toxicol,* 19**,** 767-9.

ITO, H., LI, P., KOREISHI, M., NAGATOMO, A., NISHIDA, N. & YOSHIDA, T. 2014. Ellagitannin oligomers and a neolignan from pomegranate arils and their inhibitory effects on the formation of advanced glycation end products. *Food Chemistry,* 152**,** 323-330.

IWASHIMA, Y., ETO, M., HATA, A., KAKU, K., HORIUCHI, S., USHIKUBI, F. & SANO, H. 2000. Advanced glycation end products-induced gene expression of scavenger receptors in cultured human monocyte-derived macrophages. *Biochemical and Biophysical Research Communications,* 277**,** 368-380.

JAIN, S. K. 1998. Glutathione and glucose-6-phosphate dehydrogenase deficiency can increase protein glycosylation. *Free Radic Biol Med,* 24**,** 197-201.

JAIN, S. K. & PALMER, M. 1997. The effect of oxygen radicals metabolites and vitamin E on glycosylation of proteins. *Free Radic Biol Med,* 22**,** 593-6.

JANDELEIT-DAHM, K., WATSON, A. & SORO-PAAVONEN, A. 2008. The AGE/RAGE axis in diabetes-accelerated atherosclerosis. *Clin Exp Pharmacol Physiol,* 35**,** 329-34.

JANERO, D. R. 1990. Malondialdehyde and Thiobarbituric Acid-Reactivity as Diagnostic Indexes of Lipid-Peroxidation and Peroxidative Tissue-Injury. *Free Radical Biology and Medicine,* 9**,** 515-540.

JANGA, D., PARISAEI, M. & ERSKINE, K. 2011. Revisiting instrumental vaginal delivery rates in obese pregnant women. *Archives of Disease in Childhood - Fetal and Neonatal Edition,* 96**,** Fa88.

JENKINS, D. J., KENDALL, C. W., AUGUSTIN, L. S., FRANCESCHI, S., HAMIDI, M., MARCHIE, A., JENKINS, A. L. & AXELSEN, M. 2002. Glycemic index: overview of implications in health and disease. *The American Journal of Clinical Nutrition,* 76**,** 266S-273S.

JINNO, M., TAKEUCHI, M., WATANABE, A., TERUYA, K., HIROHAMA, J., EGUCHI, N. & MIYAZAKI, A. 2011. Advanced glycation end-products accumulation compromises embryonic development and achievement of pregnancy by assisted reproductive technology. *Hum Reprod,* 26**,** 604-10.

JOGLEKAR, M. M., PANASKAR, S. N., CHOUGALE, A. D., KULKARNI, M. J. & ARVINDEKAR, A. U. 2013. A novel mechanism for antiglycative action of limonene through stabilization of protein conformation. *Molecular BioSystems,* 9**,** 2463-2472.

JOHNSON, R. B. & ONWUEGBUZIE, A. J. 2004. Mixed Methods Research: A Research Paradigm Whose Time Has Come. *Educational Researcher,* 33**,** 14-26.

JURANEK, I. & BEZEK, S. 2005. Controversy of free radical hypothesis: reactive oxygen species--cause or consequence of tissue injury? *Gen Physiol Biophys,* 24**,** 263-78.

KALOUSOVÁ, M., ZIMA, T., TESAŘ, V., DUSILOVÁ-SULKOVÁ, S. & ŠKRHA, J. 2005. Advanced glycoxidation end products in chronic diseases—clinical chemistry and genetic background. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis,* 579**,** 37-46.

KARIMI, J., GOODARZI, M. T., TAVILANI, H., KHODADADI, I. & AMIRI, I. 2012. Increased receptor for advanced glycation end products in spermatozoa of diabetic men and its association with sperm nuclear DNA fragmentation. *Andrologia,* 44 Suppl 1**,** 280-6.

KARLSEN, A., PAUR, I., BØHN, S., SAKHI, A., BORGE, G., SERAFINI, M., ERLUND, I., LAAKE, P., TONSTAD, S. & BLOMHOFF, R. 2010. Bilberry juice modulates plasma concentration of NF-κB related inflammatory markers in subjects at increased risk of CVD. *European Journal of Nutrition,* 49**,** 345-355.

KAWAGUCHI, K., MATSUMOTO, T. & KUMAZAWA, Y. 2011. Effects of antioxidant polyphenols on TNF-alpha-related diseases. *Curr Top Med Chem,* 11**,** 1767-79.

KAZEEM, M., AKANJI, M., HAFIZUR, R. M. & CHOUDHARY, M. 2012. Antiglycation, antioxidant and toxicological potential of polyphenol extracts of alligator pepper, ginger and nutmeg from Nigeria. *Asian Pac J Trop Biomed,* 2**,** 727-32.

KEANEY, J. F., LARSON, M. G., VASAN, R. S., WILSON, P. W. F., LIPINSKA, I., COREY, D., MASSARO, J. M., SUTHERLAND, P., VITA, J. A. & BENJAMIN, E. J. 2003. Obesity and Systemic Oxidative Stress: Clinical Correlates of Oxidative Stress in The Framingham Study. *Arteriosclerosis, Thrombosis, and Vascular Biology,* 23**,** 434-439.

KELLOW, N. J. & SAVIGE, G. S. 2013. Dietary advanced glycation end-product restriction for the attenuation of insulin resistance, oxidative stress and endothelial dysfunction: a systematic review. *Eur J Clin Nutr,* 67**,** 239-48.

KEMPF, K., HERDER, C., ERLUND, I., KOLB, H., MARTIN, S., CARSTENSEN, M., KOENIG, W., SUNDVALL, J., BIDEL, S., KUHA, S. & TUOMILEHTO, J. 2010. Effects of coffee consumption on subclinical inflammation and other risk factors for type 2 diabetes: a clinical trial. *Am J Clin Nutr,* 91**,** 950-7.

KENT, M. J., LIGHT, N. D. & BAILEY, A. J. 1985. Evidence for glucose-mediated covalent cross-linking of collagen after glycosylation in vitro. *Biochem J,* 225**,** 745-52.

KHAW, K. T., WAREHAM, N., BINGHAM, S., LUBEN, R., WELCH, A. & DAY, N. 2004a. Association of hemoglobin A1c with cardiovascular disease and mortality in adults: the European prospective investigation into cancer in Norfolk. *Ann Intern Med,* 141**,** 413-20.

KHAW, K. T., WAREHAM, N., BINGHAM, S., LUBEN, R., WELCH, A. & DAY, N. 2004b. Preliminary communication: glycated hemoglobin, diabetes, and incident colorectal cancer in men and women: a prospective analysis from the European prospective investigation into cancer-Norfolk study. *Cancer Epidemiol Biomarkers Prev,* 13**,** 915-9.

KIHO, T., USUI, S., HIRANO, K., AIZAWA, K. & INAKUMA, T. 2004. Tomato paste fraction inhibiting the formation of advanced glycation end-products. *Biosci Biotechnol Biochem,* 68**,** 200-5.

KIM, H. Y. & KIM, K. 2003. Protein glycation inhibitory and antioxidative activities of some plant extracts in vitro. *Journal of Agricultural and Food Chemistry,* 51**,** 1586-1591.

KIM, S. Y., DIETZ, P. M., ENGLAND, L., MORROW, B. & CALLAGHAN, W. M. 2007. Trends in Pre-pregnancy Obesity in Nine States, 1993–2003. *Obesity,* 15**,** 986-993.

KISLINGER, T., FU, C. F., HUBER, B., QU, W., TAGUCHI, A., YAN, S. D., HOFMANN, M., YAN, S. F., PISCHETSRIEDER, M., STERN, D. & SCHMIDT, A. M. 1999. N-epsilon-(carboxymethyl)lysine adducts of proteins are ligands for receptor for advanced glycation end products that activate cell signaling pathways and modulate gene expression. *Journal of Biological Chemistry,* 274**,** 31740-31749.

KLEIN, R., KLEIN, B. E., MOSS, S. E. & CRUICKSHANKS, K. J. 1994. The Wisconsin Epidemiologic Study of diabetic retinopathy. XIV. Ten-year incidence and progression of diabetic retinopathy. *Arch Ophthalmol,* 112**,** 1217-28.

KLEIN, R., KLEIN, B. E., MOSS, S. E. & CRUICKSHANKS, K. J. 1998. The Wisconsin Epidemiologic Study of Diabetic Retinopathy: XVII. The 14-year incidence and progression of diabetic retinopathy and associated risk factors in type 1 diabetes. *Ophthalmology,* 105**,** 1801-15.

KLEIN, R. L., LAIMINS, M. & LOPES-VIRELLA, M. F. 1995. Isolation, characterization, and metabolism of the glycated and nonglycated subfractions of low-density lipoproteins isolated from type I diabetic patients and nondiabetic subjects. *Diabetes,* 44**,** 1093-8.

KOENIG, R. J., BLOBSTEIN, S. H. & CERAMI, A. 1977. Structure of carbohydrate of hemoglobin AIc. *J Biol Chem,* 252**,** 2992-7.

KOENIG, R. J., PETERSON, C. M., JONES, R. L., SAUDEK, C., LEHRMAN, M. & CERAMI, A. 1976a. Correlation of glucose regulation and hemoglobin AIc in diabetes mellitus. *N Engl J Med,* 295**,** 417-20.

KOENIG, R. J., PETERSON, C. M., KILO, C., CERAMI, A. & WILLIAMSON, J. R. 1976b. Hemoglobin AIc as an indicator of the degree of glucose intolerance in diabetes. *Diabetes,* 25**,** 230-2.

KOGA, M. & KASAYAMA, S. 2010. Clinical impact of glycated albumin as another glycemic control marker. *Endocrine Journal,* 57**,** 751-762.

KOLI, R., ERLUND, I., JULA, A., MARNIEMI, J., MATTILA, P. & ALFTHAN, G. 2010. Bioavailability of Various Polyphenols from a Diet Containing Moderate Amounts of Berries†. *Journal of Agricultural and Food Chemistry,* 58**,** 3927-3932.

KONTOGIANNI, V. G., CHARISIADIS, P., MARGIANNI, E., LAMARI, F. N., GEROTHANASSIS, I. P. & TZAKOS, A. G. 2013. Olive Leaf Extracts Are a Natural Source of Advanced Glycation End Product Inhibitors. *Journal of Medicinal Food,* 16**,** 817-822.

KOYAMA, H., YAMAMOTO, H. & NISHIZAWA, Y. 2007. RAGE and soluble RAGE: potential therapeutic targets for cardiovascular diseases. *Mol Med,* 13**,** 625-35.

KRONE, C. A. & ELY, J. T. 2004. Ascorbic acid, glycation, glycohemoglobin and aging. *Med Hypotheses,* 62**,** 275-9.

KURISHITA, M., NAKASHIMA, K. & KOZU, H. 1994. A retrospective study of glucose metabolism in mothers of large babies. *Diabetes Care,* 17**,** 649-52.

KWON, J. H., KIM, Y. H., KWON, J. Y. & PARK, Y. W. 2011. Clinical significance of serum sRAGE and esRAGE in women with normal pregnancy and preeclampsia. *J Perinat Med,* 39**,** 507-13.

LANDGRAF, R. 2004. The relationship of postprandial glucose to HbA1c. *Diabetes/Metabolism Research and Reviews,* 20**,** S9-S12.

LAPOLLA, A., TRALDI, P. & FEDELE, D. 2005. Importance of measuring products of non-enzymatic glycation of proteins. *Clin Biochem,* 38**,** 103-15.

LAVIE, L. 2009. Oxidative Stress—A Unifying Paradigm in Obstructive Sleep Apnea and Comorbidities. *Progress in Cardiovascular Diseases,* 51**,** 303-312.

LEAN, M. E., ANDERSON, A. S., MORRISON, C. & CURRALL, J. 2003. Evaluation of a dietary targets monitor. *Eur J Clin Nutr,* 57**,** 667-73.

LEAN, M. E. J., HAN, T. S. & MORRISON, C. E. 1995. Waist Circumference as a Measure for Indicating Need for Weight Management. *British Medical Journal,* 311**,** 158-161.

LEDDY, M. A., POWER, M. L. & SCHULKIN, J. 2008. The impact of maternal obesity on maternal and fetal health. *Rev Obstet Gynecol,* 1**,** 170-8.

LEDL, F. & SCHLEICHER, E. 1990. New Aspects of the Maillard Reaction in Foods and in the Human-Body. *Angewandte Chemie-International Edition,* 29**,** 565-594.

LEPAPE, A., GUITTON, J. D. & MUH, J. P. 1981. Modifications of Glomerular Basement-Membrane Cross-Links in Experimental Diabetic Rats. *Biochemical and Biophysical Research Communications,* 100**,** 1214-1221.

LESGARDS, J. F., DURAND, P., LASSARRE, M., STOCKER, P., LESGARDS, G., LANTEAUME, A., PROST, M. & LEHUCHER-MICHEL, M. P. 2002. Assessment of lifestyle effects on the overall antioxidant capacity of healthy subjects. *Environ Health Perspect,* 110**,** 479-86.

LEUNG, T. Y., LEUNG, T. N., SAHOTA, D. S., CHAN, O. K., CHAN, L. W., FUNG, T. Y. & LAU, T. K. 2008. Trends in maternal obesity and associated risks of adverse pregnancy outcomes in a population of Chinese women. *BJOG: An International Journal of Obstetrics & Gynaecology,* 115**,** 1529-1537.

LI, D., DEVARAJ, S., FULLER, C., BUCALA, R. & JIALAL, I. 1996. Effect of alpha-tocopherol on LDL oxidation and glycation: in vitro and in vivo studies. *Journal of Lipid Research,* 37**,** 1978-86.

LI, K. & YANG, H. X. 2006. Value of fructosamine measurement in pregnant women with abnormal glucose tolerance. *Chin Med J (Engl),* 119**,** 1861-5.

LI, N., LIU, E., GUO, J., PAN, L., LI, B., WANG, P., LIU, J., WANG, Y., LIU, G., BACCARELLI, A. A., HOU, L. & HU, G. 2013. Maternal prepregnancy body mass index and gestational weight gain on pregnancy outcomes. *PLoS One,* 8**,** e82310.

LI, W., OTA, K., NAKAMURA, J., NARUSE, K., NAKASHIMA, E., OISO, Y. & HAMADA, Y. 2008. A BRIEF COMMUNICATION: Antiglycation Effect of Gliclazide on In Vitro AGE Formation from Glucose and Methylglyoxal. *Experimental Biology and Medicine,* 233**,** 176-179.

LICHTMAN, S. W., PISARSKA, K., BERMAN, E. R., PESTONE, M., DOWLING, H., OFFENBACHER, E., WEISEL, H., HESHKA, S., MATTHEWS, D. E. & HEYMSFIELD, S. B. 1992. Discrepancy between self-reported and actual caloric intake and exercise in obese subjects. *N Engl J Med,* 327**,** 1893-8.

LIM, E. L., HOLLINGSWORTH, K. G., ARIBISALA, B. S., CHEN, M. J., MATHERS, J. C. & TAYLOR, R. 2011. Reversal of type 2 diabetes: normalisation of beta cell function in association with decreased pancreas and liver triacylglycerol. *Diabetologia,* 54**,** 2506-14.

LIN, J., RIDKER, P. M., RIFAI, N., LEE, I. M., MANSON, J. E., BURING, J. E. & ZHANG, S. M. M. 2006. A prospective study of hemoglobin A1c concentrations and risk of breast cancer in women. *Cancer Research,* 66**,** 2869-2875.

LINDSEY, J. B., CIPOLLONE, F., ABDULLAH, S. M. & MCGUIRE, D. K. 2009. Receptor for advanced glycation end-products (RAGE) and soluble RAGE (sRAGE): cardiovascular implications. *Diabetes and Vascular Disease Research,* 6**,** 7-14.

LUDWIG, D. S. 2002. The glycemic index: Physiological mechanisms relating to obesity, diabetes, and cardiovascular disease. *JAMA,* 287**,** 2414-2423.

LUMLEY, T., DIEHR, P., EMERSON, S. & CHEN, L. 2002. The importance of the normality assumption in large public health data sets. *Annu Rev Public Health,* 23**,** 151-69.

LUNCEFORD, N. & GUGLIUCCI, A. 2005. Ilex paraguariensis extracts inhibit AGE formation more efficiently than green tea. *Fitoterapia,* 76**,** 419-427.

LYNCH, J. W., KAPLAN, G. A., COHEN, R. D., TUOMILEHTO, J. & SALONEN, J. T. 1996. Do cardiovascular risk factors explain the relation between socioeconomic status, risk of all-cause mortality, cardiovascular mortality, and acute myocardial infarction? *Am J Epidemiol,* 144**,** 934-42.

LYONS, T. J. 1993. Glycation and oxidation: a role in the pathogenesis of atherosclerosis. *Am J Cardiol,* 71**,** 26B-31B.

MACDONALD-WALLIS, C., LAWLOR, D. A., HERON, J., FRASER, A., NELSON, S. M. & TILLING, K. 2011. Relationships of risk factors for pre-eclampsia with patterns of occurrence of isolated gestational proteinuria during normal term pregnancy. *PLoS One,* 6**,** e22115.

MAGANN, E. F., DOHERTY, D. A., SANDLIN, A. T., CHAUHAN, S. P. & MORRISON, J. C. 2013. The effects of an increasing gradient of maternal obesity on pregnancy outcomes. *Aust N Z J Obstet Gynaecol,* 53**,** 250-7.

MAJOREK, K. A., POREBSKI, P. J., DAYAL, A., ZIMMERMAN, M. D., JABLONSKA, K., STEWART, A. J., CHRUSZCZ, M. & MINOR, W. 2012. Structural and immunologic characterization of bovine, horse, and rabbit serum albumins. *Mol Immunol,* 52**,** 174-82.

MAILLARD-LEFEBVRE, H., BOULANGER, E., DAROUX, M., GAXATTE, C., HUDSON, B. I. & LAMBERT, M. 2009. Soluble receptor for advanced glycation end products: a new biomarker in diagnosis and prognosis of chronic inflammatory diseases. *Rheumatology,* 48**,** 1190-1196.

MAILLARD, L. 1912. Action des acides amines sur les sucres: Formation des melanoidines par voie methodologique. *Comptes Rendus de l'Academie des Sciences,* 156**,** 148-149.

MALICKOVA, K., JAROSOVA, R., REZABEK, K., FAIT, T., MASATA, J., JANATKOVA, I., ZIMA, T. & KALOUSOVA, M. 2010. Concentrations of sRAGE in serum and follicular fluid in assisted reproductive cycles--a preliminary study. *Clin Lab,* 56**,** 377-84.

MALLIDIS, C., AGBAJE, I., ROGERS, D., GLENN, J., MCCULLOUGH, S., ATKINSON, A. B., STEGER, K., STITT, A. & MCCLURE, N. 2007. Distribution of the receptor for advanced glycation end products in the human male reproductive tract: prevalence in men with diabetes mellitus. *Hum Reprod,* 22**,** 2169-77.

MALLIDIS, C., AGBAJE, I. M., ROGERS, D. A., GLENN, J. V., PRINGLE, R., ATKINSON, A. B., STEGER, K., STITT, A. W. & MCCLURE, N. 2009. Advanced glycation end products accumulate in the reproductive tract of men with diabetes. *Int J Androl,* 32**,** 295-305.

MAMUN, A., CALLAWAY, L., O'CALLAGHAN, M., WILLIAMS, G., NAJMAN, J., ALATI, R., CLAVARINO, A. & LAWLOR, D. 2011. Associations of maternal pre-pregnancy obesity and excess pregnancy weight gains with adverse pregnancy outcomes and length of hospital stay. *BMC Pregnancy and Childbirth,* 11**,** 62.

MANACH, C., SCALBERT, A., MORAND, C., REMESY, C. & JIMENEZ, L. 2004. Polyphenols: food sources and bioavailability. *Am J Clin Nutr,* 79**,** 727-47.

MANACH, C., WILLIAMSON, G., MORAND, C., SCALBERT, A. & REMESY, C. 2005. Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *American Journal of Clinical Nutrition,* 81**,** 230S-242S.

MARANGON, K., HERBETH, B., LECOMTE, E., PAUL-DAUPHIN, A., GROLIER, P., CHANCERELLE, Y., ARTUR, Y. & SIEST, G. 1998. Diet, antioxidant status, and smoking habits in French men. *The American Journal of Clinical Nutrition,* 67**,** 231-9.

MATSUMOTO, T., TSURUMOTO, T., BABA, H., OSAKI, M., ENOMOTO, H., YONEKURA, A., SHINDO, H. & MIYATA, T. 2007. Measurement of advanced glycation endproducts in skin of patients with rheumatoid arthritis, osteoarthritis, and dialysis-related spondyloarthropathy using non-invasive methods. *Rheumatology International,* 28**,** 157-160.

MATSUSHITA, K., BLECKER, S., PAZIN, A., BERTONI, A., CHANG, P. P., CORESH, J. & SELVIN, E. 2010. The Association of Hemoglobin A1c With Incident Heart Failure Among People Without Diabetes: The Atherosclerosis Risk in Communities Study. *Diabetes,* 59**,** 2020-2026.

MATTEUCCI, E., BIASCI, E. & GIAMPIETRO, O. 2001. Advanced oxidation protein products in plasma: stability during storage and correlation with other clinical characteristics. *Acta Diabetol,* 38**,** 187-9.

MAYNE, S. T. 2003. Antioxidant Nutrients and Chronic Disease: Use of Biomarkers of Exposure and Oxidative Stress Status in Epidemiologic Research. *The Journal of Nutrition,* 133**,** 933S-940S.

MCCARTER, R. J., HEMPE, J. M., GOMEZ, R. & CHALEW, S. A. 2004. Biological variation in HbA1c predicts risk of retinopathy and nephropathy in type 1 diabetes. *Diabetes Care,* 27**,** 1259-64.

MCNAIR, E. D., WELLS, C. R., QURESHI, M., BASRAN, R., PEARCE, C., ORVOLD, J., DEVILLIERS, J. & PRASAD, K. 2010. Soluble Receptors for Advanced Glycation End Products (sRAGE) as a Predictor of Restenosis Following Percutaneous Coronary Intervention. *Clinical Cardiology,* 33**,** 678-685.

MCPHERSON, K., STEEL, C. M. & DIXON, J. M. 2000. ABC of breast diseases. Breast cancer-epidemiology, risk factors, and genetics. *BMJ,* 321**,** 624-8.

MEDINA-REMON, A., BARRIONUEVO-GONZALEZ, A., ZAMORA-ROS, R., ANDRES-LACUEVA, C., ESTRUCH, R., MARTINEZ-GONZALEZ, M. A., DIEZ-ESPINO, J. & LAMUELA-RAVENTOS, R. M. 2009. Rapid Folin-Ciocalteu method using microtiter 96-well plate cartridges for solid phase extraction to assess urinary total phenolic compounds, as a biomarker of total polyphenols intake. *Anal Chim Acta,* 634**,** 54-60.

MEDINA-REMON, A., TRESSERRA-RIMBAU, A., ARRANZ, S., ESTRUCH, R. & LAMUELA-RAVENTOS, R. M. 2012. Polyphenols excreted in urine as biomarkers of total polyphenol intake. *Bioanalysis,* 4**,** 2705-13.

MEIGS, J. B., NATHAN, D. M., D'AGOSTINO, R. B. & WILSON, P. W. F. 2002. Fasting and postchallenge glycemia and cardiovascular disease risk - The framingham offspring study. *Diabetes Care,* 25**,** 1845-1850.

MENNEN, L. I., SAPINHO, D., ITO, H., BERTRAIS, S., GALAN, P., HERCBERG, S. & SCALBERT, A. 2006. Urinary flavonoids and phenolic acids as biomarkers of intake for polyphenol-rich foods. *British Journal of Nutrition,* 96**,** 191-198.

MENZEL, E. J. & REIHSNER, R. 1996. Comparison of the effect of different inhibitors of the non-enzymatic glycation of rat tail tendons and bovine serum albumin. *Ann Clin Biochem,* 33 (Pt 3)**,** 241-8.

MINSART, A.-F., BUEKENS, P., DE SPIEGELAERE, M. & ENGLERT, Y. 2013. Neonatal outcomes in obese mothers: a population-based analysis. *BMC Pregnancy and Childbirth,* 13**,** 36.

MIYAZAKI, R., KOTANI, K., AYABE, M., TSUZAKI, K., SHIMADA, J., SAKANE, N., TAKASE, H., ICHIKAWA, H., YONEI, Y. & ISHII, K. 2013. Minor effects of green tea catechin supplementation on cardiovascular risk markers in active older people: a randomized controlled trial. *Geriatr Gerontol Int,* 13**,** 622-9.

MOHARIR, A., PECK, S. H., BUDDEN, T. & LEE, S. Y. 2013. The Role of N-Glycosylation in Folding, Trafficking, and Functionality of Lysosomal Protein CLN5. *PLoS ONE,* 8**,** e74299.

MONAMI, M., LAMANNA, C., LAMBERTUCCI, L., LONGO, R., COCCA, C., ADDANTE, F., LOTTI, E., MASOTTI, G., MARCHIONNI, N. & MANNUCCI, E. 2006. Fasting and post-prandial glycemia and their correlation with glycated hemoglobin in type 2 diabetes. *Journal of Endocrinological Investigation,* 29**,** 619-624.

MONNIER, L., LAPINSKI, H. & COLETTE, C. 2003. Contributions of fasting and postprandial plasma glucose increments to the overall diurnal hyperglycemia of type 2 diabetic patients - Variations with increasing levels of HbA(1c). *Diabetes Care,* 26**,** 881-885.

MONNIER, V. M. 1989. Toward a Maillard reaction theory of aging. *Prog Clin Biol Res,* 304**,** 1-22.

MORETTI, M., PHILLIPS, M., ABOUZEID, A., CATANEO, R. N. & GREENBERG, J. 2004. Increased breath markers of oxidative stress in normal pregnancy and in preeclampsia. *American Journal of Obstetrics and Gynecology,* 190**,** 1184-1190.

MORIARTY, S. E., SHAH, J. H., LYNN, M., JIANG, S., OPENO, K., JONES, D. P. & STERNBERG, P. 2003. Oxidation of glutathione and cysteine in human plasma associated with smoking. *Free Radic Biol Med,* 35**,** 1582-8.

MOUTON, P. R., CHACHICH, M. E., QUIGLEY, C., SPANGLER, E. & INGRAM, D. K. 2009. Caloric restriction attenuates amyloid deposition in middle-aged dtg APP/PS1 mice. *Neurosci Lett,* 464**,** 184-7.

MUELLER, G. A., MALEKI, S. J., JOHNSON, K., HURLBURT, B. K., CHENG, H., RUAN, S., NESBIT, J. B., POMES, A., EDWARDS, L. L., SCHORZMAN, A., DETERDING, L. J., PARK, H., TOMER, K. B., LONDON, R. E. & WILLIAMS, J. G. 2013. Identification of Maillard reaction products on peanut allergens that influence binding to the receptor for advanced glycation end products. *Allergy,* 68**,** 1546-1554.

MUELLER, S., RIEDEL, H. D. & STREMMEL, W. 1997. Determination of catalase activity at physiological hydrogen peroxide concentrations. *Anal Biochem,* 245**,** 55-60.

MULLEN, W., ARCHEVEQUE, M.-A., EDWARDS, C. A., MATSUMOTO, H. & CROZIER, A. 2008. Bioavailability and Metabolism of Orange Juice Flavanones in Humans: Impact of a Full-Fat Yogurt. *Journal of Agricultural and Food Chemistry,* 56**,** 11157-11164.

MUNCH, G., WESTCOTT, B., MENINI, T. & GUGLIUCCI, A. 2012. Advanced glycation endproducts and their pathogenic roles in neurological disorders. *Amino Acids,* 42**,** 1221-1236.

MURAKI, I., IMAMURA, F., MANSON, J. E., HU, F. B., WILLETT, W. C., DAM, R. M. V. & SUN, Q. 2013. Fruit consumption and risk of type 2 diabetes: results from three prospective longitudinal cohort studies. *BMJ,* 347.

MUTSAERTS, M. A., GROEN, H., BUITER-VAN DER MEER, A., SIJTSMA, A., SAUER, P. J., LAND, J. A., MOL, B. W., CORPELEIJN, E. & HOEK, A. 2014. Effects of paternal and maternal lifestyle factors on pregnancy complications and perinatal outcome. A population-based birth-cohort study: the GECKO Drenthe cohort. *Hum Reprod,* 29**,** 824-34.

MYINT, P. K., SINHA, S., WAREHAM, N. J., BINGHAM, S. A., LUBEN, R. N., WELCH, A. A. & KHAW, K. T. 2007. Glycated hemoglobin and risk of stroke in people without known diabetes in the European Prospective Investigation into Cancer (EPIC)-Norfolk prospective population study: a threshold relationship? *Stroke,* 38**,** 271-5.

MYLES, A., VISWANATH, V., SINGH, Y. P. & AGGARWAL, A. 2011. Soluble Receptor for Advanced Glycation Endproducts Is Decreased in Patients with Juvenile Idiopathic Arthritis (ERA Category) and Inversely Correlates with Disease Activity and S100A12 Levels. *Journal of Rheumatology,* 38**,** 1994-1999.

NAGASAWA, T., TABATA, N., ITO, Y., NISHIZAWA, N., AIBA, Y. & KITTS, D. D. 2003. Inhibition of glycation reaction in tissue protein incubations by water soluble rutin derivative. *Molecular and Cellular Biochemistry,* 249**,** 3-10.

NAKAMURA, T., SATO, E., FUJIWARA, N., KAWAGOE, Y., SUZUKI, T., UEDA, Y., YAMADA, S., SHOJI, H., TAKEUCHI, M., UEDA, S., MATSUI, T., ADACHI, H., OKUDA, S. & YAMAGISHI, S. 2009. Circulating levels of advanced glycation end products (AGE) and interleukin-6 (IL-6) are independent determinants of serum asymmetric dimethylarginine (ADMA) levels in patients with septic shock. *Pharmacol Res,* 60**,** 515-8.

NAMBIAR, S., VISWANATHAN, S., ZACHARIAH, B., HANUMANTHAPPA, N., AGRAWAL, A. & NADARADJAN, R. D. 2012. Possible Modulation of Glycated Protein Levels in Prehypertension by Lipid Peroxides. *Clin Exp Hypertens*.

NARDINI, M., CIRILLO, E., NATELLA, F. & SCACCINI, C. 2002. Absorption of phenolic acids in humans after coffee consumption. *J Agric Food Chem,* 50**,** 5735-41.

NARDINI, M., FORTE, M., VRHOVSEK, U., MATTIVI, F., VIOLA, R. & SCACCINI, C. 2009. White Wine Phenolics Are Absorbed and Extensively Metabolized in Humans. *Journal of Agricultural and Food Chemistry,* 57**,** 2711-2718.

NARDINI, M., NATELLA, F., SCACCINI, C. & GHISELLI, A. 2006. Phenolic acids from beer are absorbed and extensively metabolized in humans. *Journal of Nutritional Biochemistry,* 17**,** 14-22.

NARUSE, K., SADO, T., NOGUCHI, T., TSUNEMI, T., YOSHIDA, S., AKASAKA, J., KOIKE, N., OI, H. & KOBAYASHI, H. 2012. Peripheral RAGE (receptor for advanced glycation endproducts)-

ligands in normal pregnancy and preeclampsia: novel markers of inflammatory response. *J Reprod Immunol,* 93**,** 69-74.

NATHAN, D. M., KUENEN, J., BORG, R., ZHENG, H., SCHOENFELD, D., HEINE, R. J. & GRP, A. S. 2008. Translating the A1C assay into estimated average glucose values. *Diabetes Care,* 31**,** 1473-1478.

NAUDI, A., JOVE, M., AYALA, V., CASSANYE, A., SERRANO, J., GONZALO, H., BOADA, J., PRAT, J., PORTERO-OTIN, M. & PAMPLONA, R. 2012. Cellular dysfunction in diabetes as maladaptive response to mitochondrial oxidative stress. *Exp Diabetes Res,* 2012**,** 696215.

NEDIC, O., RATTAN, S. I., GRUNE, T. & TROUGAKOS, I. P. 2013. Molecular effects of advanced glycation end products on cell signalling pathways, ageing and pathophysiology. *Free Radic Res,* 47 Suppl 1**,** 28-38.

NETTLETON, J. A., HARNACK, L. J., SCRAFFORD, C. G., MINK, P. J., BARRAJ, L. M. & JACOBS, D. R., JR. 2006. Dietary flavonoids and flavonoid-rich foods are not associated with risk of type 2 diabetes in postmenopausal women. *J Nutr,* 136**,** 3039-45.

NG, Z. X., CHUA, K. H., IQBAL, T. & KUPPUSAMY, U. R. 2013. Soluble Receptor for Advanced Glycation End-Product (sRAGE)/Pentosidine Ratio: A Potential Risk Factor Determinant for Type 2 Diabetic Retinopathy. *International Journal of Molecular Sciences,* 14**,** 7480-7491.

NICHOLL, I. D. & BUCALA, R. 1998. Advanced glycation endproducts and cigarette smoking. *Cell Mol Biol (Noisy-le-grand),* 44**,** 1025-33.

NICHOLL, I. D., STITT, A. W., MOORE, J. E., RITCHIE, A. J., ARCHER, D. B. & BUCALA, R. 1998. Increased levels of advanced glycation endproducts in the lenses and blood vessels of cigarette smokers. *Mol Med,* 4**,** 594-601.

NICHOLSON, W., WANG, N. Y., BAPTISTE-ROBERTS, K., CHANG, Y. T. & POWE, N. R. 2013. Association between adiponectin and tumor necrosis factor-alpha levels at eight to fourteen weeks gestation and maternal glucose tolerance: the Parity, Inflammation, and Diabetes Study. *J Womens Health (Larchmt),* 22**,** 259-66.

NIELSEN, S. E., FREESE, R., KLEEMOLA, P. & MUTANEN, M. 2002. Flavonoids in Human Urine as Biomarkers for Intake of Fruits and Vegetables. *Cancer Epidemiology Biomarkers & Prevention,* 11**,** 459-466.

NIEMAN, D. C., GILLITT, N. D., KNAB, A. M., SHANELY, R. A., PAPPAN, K. L., JIN, F. & LILA, M. A. 2013. Influence of a Polyphenol-Enriched Protein Powder on Exercise-Induced Inflammation and Oxidative Stress in Athletes: A Randomized Trial Using a Metabolomics Approach. *Plos One,* 8.

NILSSON, P. M., LIND, L., POLLARE, T., BERNE, C. & LITHELL, H. O. 1995. Increased level of hemoglobin A1c, but not impaired insulin sensitivity, found in hypertensive and normotensive smokers. *Metabolism,* 44**,** 557-61.

NISKANEN, L., LAAKSONEN, D. E., NYYSSÖNEN, K., PUNNONEN, K., VALKONEN, V.-P., FUENTES, R., TUOMAINEN, T.-P., SALONEN, R. & SALONEN, J. T. 2004. Inflammation, Abdominal Obesity, and Smoking as Predictors of Hypertension. *Hypertension,* 44**,** 859-865.

O'BRIEN, T. E., RAY, J. G. & CHAN, W. S. 2003. Maternal body mass index and the risk of preeclampsia: A systematic overview. *Epidemiology,* 14**,** 368-374.

O'HARTE, F. P., HOJRUP, P., BARNETT, C. R. & FLATT, P. R. 1996. Identification of the site of glycation of human insulin. *Peptides,* 17**,** 1323-30.

OGAWA, S., MATSUMAE, T., KATAOKA, T., YAZAKI, Y. & YAMAGUCHI, H. 2013. Effect of acacia polyphenol on glucose homeostasis in subjects with impaired glucose tolerance: A randomized multicenter feeding trial. *Exp Ther Med,* 5**,** 1566-1572.

OLIVEIRA, L. M., LAGES, A., GOMES, R. A., NEVES, H., FAMILIA, C., COELHO, A. V. & QUINTAS, A. 2011. Insulin glycation by methylglyoxal results in native-like aggregation and inhibition of fibril formation. *BMC Biochem,* 12**,** 41.

OLIVER, E. A., BUHIMSCHI, C. S., DULAY, A. T., BAUMBUSCH, M. A., ABDEL-RAZEQ, S. S., LEE, S. Y., ZHAO, G., JING, S., PETTKER, C. M. & BUHIMSCHI, I. A. 2011. Activation of the receptor for advanced glycation end products system in women with severe preeclampsia. *J Clin Endocrinol Metab,* 96**,** 689-98.

OLIVERAS-LOPEZ, M.-J., INNOCENTI, M., MARTIN BERMUDO, F., LOPEZ-GARCIA DE LA SERRANA, H. & MULINACCI, N. 2012. Effect of extra virgin olive oil on glycaemia in healthy young subjects. *European Journal of Lipid Science and Technology,* 114**,** 999-1006.

OVASKAINEN, M.-L., TÖRRÖNEN, R., KOPONEN, J. M., SINKKO, H., HELLSTRÖM, J., REINIVUO, H. & MATTILA, P. 2008. Dietary Intake and Major Food Sources of Polyphenols in Finnish Adults. *The Journal of Nutrition,* 138**,** 562-566.

OZA-FRANK, R. & KEIM, S. A. 2013. Should obese women gain less weight in pregnancy than recommended? *Birth,* 40**,** 107-14.

PARK, S., BARRETTCONNOR, E., WINGARD, D. L., SHAN, J. & EDELSTEIN, S. 1996. GHb is a better predictor at cardiovascular disease than fasting or postchallenge plasma glucose in women without diabetes - The Rancho Bernardo Study. *Diabetes Care,* 19**,** 450-456.

PEACOCK, T. P., SHIHABI, Z. K., BLEYER, A. J., DOLBARE, E. L., BYERS, J. R., KNOVICH, M. A., CALLES-ESCANDON, J., RUSSELL, G. B. & FREEDMAN, B. I. 2008. Comparison of glycated albumin and hemoglobin A(1c) levels in diabetic subjects on hemodialysis. *Kidney Int,* 73**,** 1062-8.

PELUSO, M., MUNNIA, A., PIRO, S., JEDPIYAWONGSE, A., SANGRAJRANG, S., GIESE, R. W., CEPPI, M., BOFFETTA, P. & SRIVATANAKUL, P. 2012. Fruit and vegetable and fried food consumption and 3-(2-deoxy-beta-D-erythro-pentafuranosyl)pyrimido[1,2-alpha] purin-10(3H)-one deoxyguanosine adduct formation. *Free Radic Res,* 46**,** 85-92.

PENG, Y., YUAN, G., OVERHOLT, J. L., KUMAR, G. K. & PRABHAKAR, N. R. 2003. Systemic and cellular responses to intermittent hypoxia: evidence for oxidative stress and mitochondrial dysfunction. *Adv Exp Med Biol,* 536**,** 559-64.

PENN, L., WHITE, M., LINDSTROM, J., DEN BOER, A. T., BLAAK, E., ERIKSSON, J. G., FESKENS, E., ILANNE-PARIKKA, P., KEINANEN-KIUKAANNIEMI, S. M., WALKER, M., MATHERS, J. C., UUSITUPA, M. & TUOMILEHTO, J. 2013. Importance of weight loss maintenance and risk prediction in the prevention of type 2 diabetes: analysis of European Diabetes Prevention Study RCT. *PLoS One,* 8**,** e57143.

PEREZ-JIMENEZ, J., HUBERT, J., HOOPER, L., CASSIDY, A., MANACH, C., WILLIAMSON, G. & SCALBERT, A. 2010. Urinary metabolites as biomarkers of polyphenol intake in humans: a systematic review. *American Journal of Clinical Nutrition,* 92**,** 801-809.

PHAM-HUY, L. A., HE, H. & PHAM-HUY, C. 2008. Free radicals, antioxidants in disease and health. *Int J Biomed Sci,* 4**,** 89-96.

PLUTZKY, J. 2011. Macrovascular effects and safety issues of therapies for type 2 diabetes. *Am J Cardiol,* 108**,** 25B-32B.

POPPITT, S. D., SWANN, D., BLACK, A. E. & PRENTICE, A. M. 1998. Assessment of selective under-reporting of food intake by both obese and non-obese women in a metabolic facility. *Int J Obes Relat Metab Disord,* 22**,** 303-11.

PORTA, M., SJOELIE, A. K., CHATURVEDI, N., STEVENS, L., ROTTIERS, R., VEGLIO, M., FULLER, J. H. & GROUP, E. P. C. S. 2001. Risk factors for progression to proliferative diabetic retinopathy in the EURODIAB Prospective Complications Study. *Diabetologia,* 44**,** 2203-9.

POSTON, L., BRILEY, A. L., BARR, S., BELL, R., CROKER, H., COXON, K., ESSEX, H. N., HUNT, C., HAYES, L., HOWARD, L. M., KHAZAEZADEH, N., KINNUNEN, T., NELSON, S. M., OTENG-NTIM, E., ROBSON, S. C., SATTAR, N., SEED, P. T., WARDLE, J., SANDERS, T. A. & SANDALL, J. 2013. Developing a complex intervention for diet and activity behaviour change in obese pregnant women (the UPBEAT trial); assessment of behavioural change and process evaluation in a pilot randomised controlled trial. *BMC Pregnancy Childbirth,* 13**,** 148.

POU, K. M., MASSARO, J. M., HOFFMANN, U., VASAN, R. S., MAUROVICH-HORVAT, P., LARSON, M. G., KEANEY, J. F., MEIGS, J. B., LIPINSKA, I., KATHIRESAN, S., MURABITO, J. M., O'DONNELL, C. J., BENJAMIN, E. J. & FOX, C. S. 2007. Visceral and Subcutaneous Adipose Tissue Volumes Are Cross-Sectionally Related to Markers of Inflammation and Oxidative Stress: The Framingham Heart Study. *Circulation,* 116**,** 1234-1241.

PRADHAN, A. D., RIFAI, N., BURING, J. E. & RIDKER, P. M. 2007. Hemoglobin a1c predicts diabetes but not cardiovascular disease in nondiabetic women. *American Journal of Medicine,* 120**,** 720-727.

PUTT, S. N. H., SHAW, A. P. M., WOODS, A. J., TYLER, L. & JAMES, A. D. 1987. Veterinary epidemiology and economics in Africa. A manual for use in the design and appraisal of livestock health policy.

QING, Z., LING-LING, E., DONG-SHENG, W. & HONG-CHEN, L. 2012. Relationship of advanced oxidative protein products in human saliva and plasma: age- and gender-related changes and stability during storage. *Free Radical Research,* 46**,** 1201-1206.

RAIJMAKERS, M. T. M., DECHEND, R. & POSTON, L. 2004. Oxidative Stress and Preeclampsia: Rationale for Antioxidant Clinical Trials. *Hypertension,* 44**,** 374-380.

RAMACHENDERAN, J., BRADFORD, J. & MCLEAN, M. 2008. Maternal obesity and pregnancy complications: A review. *Australian and New Zealand Journal of Obstetrics and Gynaecology,* 48**,** 228-235.

RAMASAMY, R., VANNUCCI, S. J., YAN, S. S., HEROLD, K., YAN, S. F. & SCHMIDT, A. M. 2005. Advanced glycation end products and RAGE: a common thread in aging, diabetes, neurodegeneration, and inflammation. *Glycobiology,* 15**,** 16R-28R.

RASMUSSEN, S., IRGENS, L. & ESPINOZA, J. 2014. Maternal obesity and excess of fetal growth in pre-eclampsia. *Bjog*.

RAUCCI, A., CUGUSI, S., ANTONELLI, A., BARABINO, S., BIERHAUS, A., SAFTIG, P. & BIANCHI, M. E. 2008. A soluble form of RAGE is produced by proteolytic cleavage of the membrane bound form by the sheddase a disintegrin and metalloprotease 10 (ADAM10). *Journal of Leukocyte Biology,* 84**,** A10-A10.

RAY, J. G., VERMEULEN, M. J., SHAPIRO, J. L. & KENSHOLE, A. B. 2001. Maternal and neonatal outcomes in pregestational and gestational diabetes mellitus, and the influence of maternal obesity and weight gain: the DEPOSIT study. *QJM,* 94**,** 347-356.

REISER, K. M. 1994. Influence of Age and Long-Term Dietary Restriction on Enzymatically Mediated Crosslinks and Nonenzymatic Glycation of Collagen in Mice. *Journal of Gerontology,* 49**,** B71-B79.

RIZZO, A. M., BERSELLI, P., ZAVA, S., MONTORFANO, G., NEGRONI, M., CORSETTO, P. & BERRA, B. 2010. Endogenous antioxidants and radical scavengers. *Adv Exp Med Biol,* 698**,** 52-67.

RODRIGO, R., MIRANDA, A. & VERGARA, L. 2011. Modulation of endogenous antioxidant system by wine polyphenols in human disease. *Clinica Chimica Acta,* 412**,** 410-424.

ROMERO, R., GOTSCH, F., PINELES, B. & KUSANOVIC, J. P. 2007. Inflammation in pregnancy: its roles in reproductive physiology, obstetrical complications, and fetal injury. *Nutr Rev,* 65**,** S194-202.

RONDEAU, P. & BOURDON, E. 2011. The glycation of albumin: structural and functional impacts. *Biochimie,* 93**,** 645-58.

ROOHK, H. V. & ZAIDI, A. R. 2008. A review of glycated albumin as an intermediate glycation index for controlling diabetes. *J Diabetes Sci Technol,* 2**,** 1114-21.

ROOS, N., KIELER, H., SAHLIN, L., EKMAN-ORDEBERG, G., FALCONER, H. & STEPHANSSON, O. 2011. Risk of adverse pregnancy outcomes in women with polycystic ovary syndrome: population based cohort study. *Bmj,* 343**,** d6309.

ROTHWELL, J. A., URPI-SARDA, M., BOTO-ORDONEZ, M., KNOX, C., LLORACH, R., EISNER, R., CRUZ, J., NEVEU, V., WISHART, D., MANACH, C., ANDRES-LACUEVA, C. & SCALBERT, A. 2012. Phenol-Explorer 2.0: a major update of the Phenol-Explorer database integrating data on polyphenol metabolism and pharmacokinetics in humans and experimental animals. *Database (Oxford),* 2012**,** bas031.

ROURA, E., ANDRÉS-LACUEVA, C., ESTRUCH, R. & LAMUELA-RAVENTÓS, R. M. 2006. Total Polyphenol Intake Estimated by a Modified Folin–Ciocalteu Assay of Urine. *Clinical Chemistry,* 52**,** 749-752.

SABANAYAGAM, C., LIEW, G., TAI, E. S., SHANKAR, A., LIM, S. C., SUBRAMANIAM, T. & WONG, T. Y. 2009. Relationship between glycated haemoglobin and microvascular complications: Is there a natural cut-off point for the diagnosis of diabetes? *Diabetologia,* 52**,** 1279-1289.

SABATER, J., QUEREDA, C., HERRERA, I., PASCUAL, J., VILLAFRUELA, J. J. & ORTUNO, J. 1991. Nonenzymatic Glycosylation of Hemoglobin and Total Plasmatic Proteins in End-Stage Renal-Disease. *American Journal of Nephrology,* 11**,** 37-43.

SADO, T., NARUSE, K., NOGUCHI, T., HARUTA, S., YOSHIDA, S., TANASE, Y., KITANAKA, T., OI, H. & KOBAYASHI, H. 2011. Inflammatory pattern recognition receptors and their ligands: factors contributing to the pathogenesis of preeclampsia. *Inflamm Res,* 60**,** 509-20.

SAHYOUN, N. R., ANDERSON, A. L., KANAYA, A. M., KOH-BANERJEE, P., KRITCHEVSKY, S. B., DE REKENEIRE, N., TYLAVSKY, F. A., SCHWARTZ, A. V., LEE, J. S. & HARRIS, T. B. 2005. Dietary glycemic index and load, measures of glucose metabolism, and body fat distribution in older adults. *The American Journal of Clinical Nutrition,* 82**,** 547-552.

SAKURAI, M., SAITOH, S., MIURA, K., NAKAGAWA, H., OHNISHI, H., AKASAKA, H., KADOTA, A., KITA, Y., HAYAKAWA, T., OHKUBO, T., OKAYAMA, A., OKAMURA, T., UESHIMA, H. & GROUP, N. D. R. 2013. HbA1c and the risks for all-cause and cardiovascular mortality in the general Japanese population: NIPPON DATA90. *Diabetes Care,* 36**,** 3759-65.

SAMAHA, F. F., IQBAL, N., SESHADRI, P., CHICANO, K. L., DAILY, D. A., MCGRORY, J., WILLIAMS, T., WILLIAMS, M., GRACELY, E. J. & STERN, L. 2003. A Low-Carbohydrate as Compared with a Low-Fat Diet in Severe Obesity. *New England Journal of Medicine,* 348**,** 2074-2081.

SARGEANT, L. A., WAREHAM, N. J., BINGHAM, S., DAY, N. E., LUBEN, R. N., OAKES, S., WELCH, A. & KHAW, K. T. 2000. Vitamin C and hyperglycemia in the European Prospective Investigation into Cancer--Norfolk (EPIC-Norfolk) study: a population-based study. *Diabetes Care,* 23**,** 726-732.

SCALBERT, A., MORAND, C., MANACH, C. & RÉMÉSY, C. 2002. Absorption and metabolism of polyphenols in the gut and impact on health. *Biomedicine & Pharmacotherapy,* 56**,** 276-282.

SCALBERT, A. & WILLIAMSON, G. 2000. Dietary intake and bioavailability of polyphenols. *Journal of Nutrition,* 130**,** 2073s-2085s.

SCHLEICHER, E. D., OLGEMÖLLER, B., WIEDENMANN, E. & GERBITZ, K. D. 1993. Specific glycation of albumin depends on its half-life. *Clinical Chemistry,* 39**,** 625-8.

SCHMIDT, A. M., HORI, O., BRETT, J., YAN, S. D., WAUTIER, J. L. & STERN, D. 1994. Cellular receptors for advanced glycation end products. Implications for induction of oxidant stress and cellular dysfunction in the pathogenesis of vascular lesions. *Arterioscler Thromb,* 14**,** 1521-8.

SCHMIDT, A. M., HORI, O., CHEN, J. X., LI, J. F., CRANDALL, J., ZHANG, J., CAO, R., YAN, S. D., BRETT, J. & STERN, D. 1995. Advanced glycation endproducts interacting with their endothelial receptor induce expression of vascular cell adhesion molecule-1 (VCAM-1) in cultured human endothelial cells and in mice. A potential mechanism for the accelerated vasculopathy of diabetes. *J Clin Invest,* 96**,** 1395-403.

SCHMIDT, A. M., VIANNA, M., GERLACH, M., BRETT, J., RYAN, J., KAO, J., ESPOSITO, C., HEGARTY, H., HURLEY, W., CLAUSS, M. & ET AL. 1992. Isolation and characterization of two binding proteins for advanced glycosylation end products from bovine lung which are present on the endothelial cell surface. *J Biol Chem,* 267**,** 14987-97.

SCHMIDT, A. M., YAN, S. D., YAN, S. F. & STERN, D. M. 2000. The biology of the receptor for advanced glycation end products and its ligands. *Biochim Biophys Acta,* 1498**,** 99-111.

SCHMIDT, A. M., YAN, S. D., YAN, S. F. & STERN, D. M. 2001. The multiligand receptor RAGE as a progression factor amplifying immune and inflammatory responses. *The Journal of Clinical Investigation,* 108**,** 949-955.

SCHULTZE, H. E. & HEREMANS, J. F. 1966. *Molecular biology of human proteins : with special reference to plasma proteins,* Amsterdam ; London, Elsevier.

SCHWARZE, P. E., OVREVIK, J., LAG, M., REFSNES, M., NAFSTAD, P., HETLAND, R. B. & DYBING, E. 2006. Particulate matter properties and health effects: consistency of epidemiological and toxicological studies. *Hum Exp Toxicol,* 25**,** 559-79.

SCOTTISH GOVERNMENT. 2013. *Design and Content* [Online]. Scottish Government, St. Andrew's House, Regent Road, Edinburgh EH1 3DG Tel:0131 556 8400 ceu@scotland.gsi.gov.uk. Available: http://www.scotland.gov.uk/Topics/Statistics/Browse/Health/scottish-health-survey/SurveyDesignContent#samplesize.

SCOTTISH GOVERNMENT. 2014. *Scottish Health Survey* [Online]. Scottish Government, St. Andrew's House, Regent Road, Edinburgh EH1 3DG Tel:0131 556 8400

ceu@scotland.gsi.gov.uk. Available: http://www.scotland.gov.uk/Topics/Statistics/Browse/Health/scottish-health-survey.

SEBEKOVA, K., KRAJCOVIOVA-KUDLACKOVA, M., SCHINZEL, R., FAIST, V., KLVANOVA, J. & HEIDLAND, A. 2001. Plasma levels of advanced glycation end products in healthy, long-term vegetarians and subjects on a western mixed diet. *Eur J Nutr,* 40**,** 275-81.

ŠEBEKOVÁ, K. & SOMOZA, V. 2007. Dietary advanced glycation endproducts (AGEs) and their health effects – PRO. *Molecular Nutrition & Food Research,* 51**,** 1079-1084.

SELL, D. R., LANE, M. A., OBRENOVICH, M. E., MATTISON, J. A., HANDY, A., INGRAM, D. K., CUTLER, R. G., ROTH, G. S. & MONNIER, V. M. 2003. The effect of caloric restriction on glycation and glycoxidation in skin collagen of nonhuman primates. *J Gerontol A Biol Sci Med Sci,* 58**,** 508-16.

SELL, D. R. & MONNIER, V. M. 1989a. Isolation, purification and partial characterization of novel fluorophores from aging human insoluble collagen-rich tissue. *Connect Tissue Res,* 19**,** 77-92.

SELL, D. R. & MONNIER, V. M. 1989b. Structure elucidation of a senescence cross-link from human extracellular matrix. Implication of pentoses in the aging process. *J Biol Chem,* 264**,** 21597-602.

SELVARAJ, N., BOBBY, Z., KONER, B. C. & DAS, A. K. 2005. Reassessing the increased glycation of hemoglobin in nondiabetic chronic renal failure patients: A hypothesis on the role of lipid peroxides. *Clinica Chimica Acta,* 360**,** 108-113.

SELVARAJ, N., BOBBY, Z. & SRIDHAR, M. G. 2008. Oxidative stress: Does it play a role in the genesis of early glycated proteins? *Medical Hypotheses,* 70**,** 265-268.

SELVIN, E., CORESH, J., SHAHAR, E., ZHANG, L., STEFFES, M. & SHARRETT, A. R. 2005. Glycaemia (haemoglobin A1c) and incident ischaemic stroke: the Atherosclerosis Risk in Communities (ARIC) Study. *Lancet Neurol,* 4**,** 821-6.

SELVIN, E., HALUSHKA, M. K., RAWLINGS, A. M., HOOGEVEEN, R. C., BALLANTYNE, C. M., CORESH, J. & ASTOR, B. C. 2013. sRAGE and risk of diabetes, cardiovascular disease, and death. *Diabetes,* 62**,** 2116-21.

SELVIN, E., RAWLINGS, A. M., GRAMS, M., KLEIN, R., SHARRETT, A. R., STEFFES, M. & CORESH, J. 2014. Fructosamine and glycated albumin for risk stratification and prediction of incident diabetes and microvascular complications: a prospective cohort analysis of the Atherosclerosis Risk in Communities (ARIC) study. *The Lancet Diabetes & Endocrinology*.

SELVIN, E., STEFFES, M. W., BALLANTYNE, C. M., HOOGEVEEN, R. C., CORESH, J. & BRANCATI, F. L. 2011. Racial differences in glycemic markers: a cross-sectional analysis of community-based data. *Ann Intern Med,* 154**,** 303-9.

SELVIN, E., STEFFES, M. W., ZHU, H., MATSUSHITA, K., WAGENKNECHT, L., PANKOW, J., CORESH, J. & BRANCATI, F. L. 2010. Glycated hemoglobin, diabetes, and cardiovascular risk in nondiabetic adults. *N Engl J Med,* 362**,** 800-11.

SENSI, M., TANZI, P., BRUNO, M. R., MANCUSO, M. & ANDREANI, D. 1986. Human Glomerular-Basement-Membrane - Altered Binding Characteristics Following Invitro Nonenzymatic Glycosylation. *Annals of the New York Academy of Sciences,* 488**,** 549-552.

SENSI, M., TANZI, P., BRUNO, M. R., POZZILLI, P., MANCUSO, M., GAMBARDELLA, S. & DI MARIO, U. 1989. Nonenzymic glycation of isolated human glomerular basement membrane changes its physicochemical characteristics and binding properties. *Nephron,* 52**,** 222-6.

SERAFINI, M. & DEL RIO, D. 2004. Understanding the association between dietary antioxidants, redox status and disease: is the Total Antioxidant Capacity the right tool? *Redox Rep,* 9**,** 145-52.

SERAFINI, M., TESTA, M. F., VILLAÑO, D., PECORARI, M., VAN WIEREN, K., AZZINI, E., BRAMBILLA, A. & MAIANI, G. 2009. Antioxidant activity of blueberry fruit is impaired by association with milk. *Free Radical Biology and Medicine,* 46**,** 769-774.

SEVERIN, F. F., FENIOUK, B. A. & SKULACHEV, V. P. 2013. Advanced glycation of cellular proteins as a possible basic component of the "master biological clock". *Biochemistry (Moscow),* 78**,** 1043-1047.

SHAKLAI, N., GARLICK, R. L. & BUNN, H. F. 1984. Nonenzymatic glycosylation of human serum albumin alters its conformation and function. *J Biol Chem,* 259, 3812-7.

SHARMA, J. B., SHARMA, A., BAHADUR, A., VIMALA, N., SATYAM, A. & MITTAL, S. 2006. Oxidative stress markers and antioxidant levels in normal pregnancy and pre-eclampsia. *International Journal of Gynecology & Obstetrics,* 94**,** 23-27.

SHIINA, Y., FUNABASHI, N., LEE, K., MURAYAMA, T., NAKAMURA, K., WAKATSUKI, Y., DAIMON, M. & KOMURO, I. 2009. Acute effect of oral flavonoid-rich dark chocolate intake on coronary circulation, as compared with non-flavonoid white chocolate, by transthoracic Doppler echocardiography in healthy adults. *Int J Cardiol,* 131**,** 424-9.

SIES, H., STAHL, W. & SEVANIAN, A. 2005. Nutritional, Dietary and Postprandial Oxidative Stress. *The Journal of Nutrition,* 135**,** 969-972.

SIMON, D., SENAN, C., GARNIER, P., SAINT-PAUL, M. & PAPOZ, L. 1989. Epidemiological features of glycated haemoglobin A1c-distribution in a healthy population. The Telecom Study. *Diabetologia,* 32**,** 864-9.

SINCLAIR, D. A. 2005. Toward a unified theory of caloric restriction and longevity regulation. *Mech Ageing Dev,* 126**,** 987-1002.

SINGLETON, V. L. & ROSSI, J. A. 1965. Colorimetry of Total Phenolics with Phosphomolybdic-Phosphotungstic Acid Reagents. *American Journal of Enology and Viticulture,* 16**,** 144-158.

SKYLER, J. S., BERGENSTAL, R., BONOW, R. O., BUSE, J., DEEDWANIA, P., GALE, E. A., HOWARD, B. V., KIRKMAN, M. S., KOSIBOROD, M., REAVEN, P., SHERWIN, R. S., AMERICAN DIABETES, A., AMERICAN COLLEGE OF CARDIOLOGY, F. & AMERICAN HEART, A. 2009. Intensive glycemic control and the prevention of cardiovascular events: implications of the ACCORD, ADVANCE, and VA Diabetes Trials: a position statement of the American Diabetes Association and a Scientific Statement of the American College of Cardiology Foundation and the American Heart Association. *J Am Coll Cardiol,* 53**,** 298-304.

SLAVIN, J. 2003. Why whole grains are protective: biological mechanisms. *Proceedings of the Nutrition Society,* 62**,** 129-134.

SMITH, M. A., SAYRE, L. M., MONNIER, V. M. & PERRY, G. 1995. Radical AGEing in Alzheimer's disease. *Trends Neurosci,* 18**,** 172-6.

SOMPONG, W., MEEPROM, A., CHENG, H. & ADISAKWATTANA, S. 2013. A Comparative Study of Ferulic Acid on Different Monosaccharide-Mediated Protein Glycation and Oxidative Damage in Bovine Serum Albumin. *Molecules,* 18**,** 13886-13903.

SONG, Y., MANSON, J. E., BURING, J. E., SESSO, H. D. & LIU, S. 2005. Associations of dietary flavonoids with risk of type 2 diabetes, and markers of insulin resistance and systemic inflammation in women: a prospective study and cross-sectional analysis. *J Am Coll Nutr,* 24**,** 376-84.

SOULIMANE, S., SIMON, D., HERMAN, W., LANGE, C., LEE, C. Y., COLAGIURI, S., SHAW, J., ZIMMET, P., MAGLIANO, D., FERREIRA, S. G., DONG, Y., ZHANG, L., JORGENSEN, T., TUOMILEHTO, J., MOHAN, V., CHRISTENSEN, D., KADUKA, L., DEKKER, J., NIJPELS, G., STEHOUWER, C. A., LANTIERI, O., FUJIMOTO, W., LEONETTI, D., MCNEELY, M., BORCH-JOHNSEN, K., BOYKO, E., VISTISEN, D. & BALKAU, B. 2014. HbA1c, fasting and 2 h plasma glucose in current, ex- and never-smokers: a meta-analysis. *Diabetologia,* 57**,** 30-39.

SOUSA, M. M., YAN, S. D., STERN, D. & SARAIVA, M. J. 2000. Interaction of the receptor for advanced glycation end products (RAGE) with transthyretin triggers nuclear transcription factor kB (NF-kB) activation. *Lab Invest,* 80**,** 1101-10.

SPANOS, G. A., WROLSTAD, R. E. & HEATHERBELL, D. A. 1990. Influence of Processing and Storage on the Phenolic Composition of Apple Juice. *Journal of Agricultural and Food Chemistry,* 38**,** 1572-1579.

SPENCER, J. P., ABD EL MOHSEN, M. M., MINIHANE, A. M. & MATHERS, J. C. 2008. Biomarkers of the intake of dietary polyphenols: strengths, limitations and application in nutrition research. *Br J Nutr,* 99**,** 12-22.

SPIRO, R. G. 2002. Protein glycosylation: nature, distribution, enzymatic formation, and disease implications of glycopeptide bonds. *Glycobiology,* 12**,** 43R-56R.

SRIKANTH, V., MACZUREK, A., PHAN, T., STEELE, M., WESTCOTT, B., JUSKIW, D. & MUNCH, G. 2011. Advanced glycation endproducts and their receptor RAGE in Alzheimer's disease. *Neurobiol Aging,* 32**,** 763-77.

STENSEN, M. H., TANBO, T., STORENG, R. & FEDORCSAK, P. 2014. Advanced glycation end products and their receptor contribute to ovarian ageing. *Human Reproduction,* 29**,** 125-134.

STEPHENS, N. G., PARSONS, A., SCHOFIELD, P. M., KELLY, F., CHEESEMAN, K. & MITCHINSON, M. J. 1996. Randomised controlled trial of vitamin E in patients with coronary disease: Cambridge Heart Antioxidant Study (CHAOS). *Lancet,* 347**,** 781-6.

STITT, A. W. 2001. Advanced glycation: an important pathological event in diabetic and age related ocular disease. *British Journal of Ophthalmology,* 85**,** 746-753.

STOTE, K. S. & BAER, D. J. 2008. Tea consumption may improve biomarkers of insulin sensitivity and risk factors for diabetes. *J Nutr,* 138**,** 1584S-1588S.

STRACKE, B. A., RUEFER, C. E., BUB, A., SEIFERT, S., WEIBEL, F. P., KUNZ, C. & WATZL, B. 2010. No effect of the farming system (organic/conventional) on the bioavailability of apple (Malus domestica Bork., cultivar Golden Delicious) polyphenols in healthy men: a comparative study. *European Journal of Nutrition,* 49**,** 301-310.

STRATTON, I. M., ADLER, A. I., NEIL, H. A., MATTHEWS, D. R., MANLEY, S. E., CULL, C. A., HADDEN, D., TURNER, R. C. & HOLMAN, R. R. 2000. Association of glycaemia with macrovascular and microvascular complications of type 2 diabetes (UKPDS 35): prospective observational study. *BMJ,* 321**,** 405-12.

SWEET, M. 2011. Deaths from chronic disease fall in Australia but concerns rise for the future. *BMJ,* 342.

SYDSJO, G., SYDSJO, A., BRYNHILDSEN, J. & JOSEFSSON, A. 2010. Trends in caesarean section and instrumental deliveries in relation to Body Mass Index: a clinical survey during 1978 - 2001. *Reprod Health,* 7**,** 18.

TAKAHASHI, S., UCHINO, H., SHIMIZU, T., KANAZAWA, A., TAMURA, Y., SAKAI, K., WATADA, H., HIROSE, T., KAWAMORI, R. & TANAKA, Y. 2007. Comparison of glycated albumin (GA) and glycated hemoglobin (HbA1c) in type 2 diabetic patients: Usefulness of GA for evaluation of short-term changes in glycemic control. *Endocrine Journal,* 54**,** 139-144.

TAKEUCHI, M. & YAMAGISHI, S. 2008. Possible involvement of advanced glycation end-products (AGEs) in the pathogenesis of Alzheimer's disease. *Curr Pharm Des,* 14**,** 973-8.

TANAKA, S., AVIGAD, G., BRODSKY, B. & EIKENBERRY, E. F. 1988. Glycation induces expansion of the molecular packing of collagen. *J Mol Biol,* 203**,** 495-505.

TANJI, N., MARKOWITZ, G. S., FU, C. F., KISLINGER, T., TAGUCHI, A., PISCHETSRIEDER, M., STERN, D., SCHMIDT, A. M. & D'AGATI, V. D. 2000. Expression of advanced glycation end products and their cellular receptor RAGE in diabetic nephropathy and nondiabetic renal disease. *Journal of the American Society of Nephrology,* 11**,** 1656-1666.

TARWADI, K. & AGTE, V. 2011. Effect of Micronutrients on Methylglyoxal-Mediated In Vitro Glycation of Albumin. *Biological Trace Element Research,* 143**,** 717-725.

TAYEH, N., RUNGASSAMY, T. & ALBANI, J. R. 2009. Fluorescence spectral resolution of tryptophan residues in bovine and human serum albumins. *J Pharm Biomed Anal,* 50**,** 107-16.

TAYLOR, A., LIPMAN, R. D., JAHNGEN-HODGE, J., PALMER, V., SMITH, D., PADHYE, N., DALLAL, G. E., CYR, D. E., LAXMAN, E., SHEPARD, D. & ET AL. 1995. Dietary calorie restriction in the Emory mouse: effects on lifespan, eye lens cataract prevalence and progression, levels of ascorbate, glutathione, glucose, and glycohemoglobin, tail collagen breaktime, DNA and RNA oxidation, skin integrity, fecundity, and cancer. *Mech Ageing Dev,* 79**,** 33-57.

TENENBAUM-GAVISH, K. & HOD, M. 2013. Impact of maternal obesity on fetal health. *Fetal Diagn Ther,* 34**,** 1-7.

TERRY, T., RARAVIKAR, K., CHOKRUNGVARANON, N. & REAVEN, P. D. 2012. Does aggressive glycemic control benefit macrovascular and microvascular disease in type 2 diabetes? Insights from ACCORD, ADVANCE, and VADT. *Curr Cardiol Rep,* 14**,** 79-88.

THOME, J., MÜNCH, G., MÜLLER, R., SCHINZEL, R., KORNHUBER, J., BLUM-DEGEN, D., SITZMANN, L., RÖSLER, M., HEIDLAND, A. & RIEDERER, P. 1996. Advanced glycation endproducts —

associated parameters in the peripheral blood of patients with Alzheimer's disease. *Life Sciences,* 59**,** 679-685.

THOMPSON F. E., S. A. F. 2008. *Nutrition in the Prevention and Treatment of Disease*.

THORNALLEY, P., WOLFF, S., CRABBE, J. & STERN, A. 1984. The autoxidation of glyceraldehyde and other simple monosaccharides under physiological conditions catalysed by buffer ions. *Biochim Biophys Acta,* 797**,** 276-87.

THORNALLEY, P. J. 2003a. The enzymatic defence against glycation in health, disease and therapeutics: a symposium to examine the concept. *Biochem Soc Trans,* 31**,** 1341-2.

THORNALLEY, P. J. 2003b. Glyoxalase I--structure, function and a critical role in the enzymatic defence against glycation. *Biochem Soc Trans,* 31**,** 1343-8.

THORNALLEY, P. J. 2003c. Use of aminoguanidine (Pimagedine) to prevent the formation of advanced glycation endproducts. *Archives of Biochemistry and Biophysics,* 419**,** 31-40.

THORPE, S. R. & BAYNES, J. W. 2003. Maillard reaction products in tissue proteins: New products and new perspectives. *Amino Acids,* 25**,** 275-281.

TOESCU, V., NUTTALL, S. L., MARTIN, U., KENDALL, M. J. & DUNNE, F. 2002. Oxidative stress and normal pregnancy. *Clinical Endocrinology,* 57**,** 609-613.

TUCKER, K. L. 2007. Assessment of usual dietary intake in population studies of gene–diet interaction. *Nutrition, Metabolism and Cardiovascular Diseases,* 17**,** 74-81.

TURK, Z., LJUBIC, S., TURK, N. & BENKO, B. 2001. Detection of autoantibodies against advanced glycation endproducts and AGE-immune complexes in serum of patients with diabetes mellitus. *Clinica Chimica Acta,* 303**,** 105-115.

URIBARRI, J., CAI, W., SANDU, O., PEPPA, M., GOLDBERG, T. & VLASSARA, H. 2005. Diet-Derived Advanced Glycation End Products Are Major Contributors to the Body's AGE Pool and Induce Inflammation in Healthy Subjects. *Annals of the New York Academy of Sciences,* 1043**,** 461-466.

URIBARRI, J., WOODRUFF, S., GOODMAN, S., CAI, W., CHEN, X., PYZIK, R., YONG, A., STRIKER, G. E. & VLASSARA, H. 2010. Advanced Glycation End Products in Foods and a Practical Guide to Their Reduction in the Diet. *Journal of the American Dietetic Association,* 110**,** 911-916.e12.

URIOS, P., GRIGOROVA-BORSOS, A. M. & STERNBERG, M. 2007. Aspirin inhibits the formation of pentosidine, a cross-linking advanced glycation end product, in collagen. *Diabetes Res Clin Pract,* 77**,** 337-40.

URPI-SARDA, M., MONAGAS, M., KHAN, N., LLORACH, R., MA LAMUELA-RAVENTOS, R., JAUREGUI, O., ESTRUCH, R., IZQUIERDO-PULIDO, M. & ANDRES-LACUEVA, C. 2009. Targeted metabolic profiling of phenolics in urine and plasma after regular consumption of cocoa by liquid chromatography-tandem mass spectrometry. *Journal of Chromatography A,* 1216**,** 7258-7267.

VAN'T RIET, E., RIJKELIJKHUIZEN, J. M., ALSSEMA, M., NIJPELS, G., STEHOUWER, C. D. A., HEINE, R. J. & DEKKER, J. M. 2012. HbA1c is an independent predictor of non-fatal cardiovascular disease in a Caucasian population without diabetes: a 10-year follow-up of the Hoorn Study. *European Journal of Preventive Cardiology,* 19**,** 23-31.

VARGAS, H. O., NUNES, S. O. V., DE CASTRO, M. R. P., VARGAS, M. M., BARBOSA, D. S., BORTOLASCI, C. C., VENUGOPAL, K., DODD, S. & BERK, M. 2013. Oxidative stress and inflammatory markers are associated with depression and nicotine dependence. *Neuroscience Letters,* 544**,** 136-140.

VARMA, S. D. & DEVAMANOHARAN, P. S. 1991. Hydrogen peroxide in human blood. *Free Radic Res Commun,* 14**,** 125-31.

VAZZANA, N., SANTILLI, F., CUCCURULLO, C. & DAVÌ, G. 2009. Soluble forms of RAGE in internal medicine. *Internal and Emergency Medicine,* 4**,** 389-401.

VEERANNA, V., RAMESH, K., ZALAWADIYA, S. K., NIRAJ, A., PRADHAN, J., JACOB, S. & AFONSO, L. 2011. Glycosylated hemoglobin and prevalent metabolic syndrome in nondiabetic multiethnic U.S. adults. *Metab Syndr Relat Disord,* 9**,** 361-7.

VENKATRAMAN, J., AGGARWAL, K. & BALARAM, P. 2001. Helical peptide models for protein glycation: proximity effects in catalysis of the Amadori rearrangement. *Chemistry & Biology,* 8**,** 611-625.

VERZELLONI, E., PELLACANI, C., TAGLIAZUCCHI, D., TAGLIAFERRI, S., CALANI, L., COSTA, L. G., BRIGHENTI, F., BORGES, G., CROZIER, A., CONTE, A. & DEL RIO, D. 2011a. Antiglycative and neuroprotective activity of colon-derived polyphenol catabolites. *Mol Nutr Food Res,* 55 Suppl 1**,** S35-43.

VERZELLONI, E., TAGLIAZUCCHI, D., DEL RIO, D., CALANI, L. & CONTE, A. 2011b. Antiglycative and antioxidative properties of coffee fractions. *Food Chemistry,* 124**,** 1430-1435.

VINSON, J. A., DEMKOSKY, C. A., NAVARRE, D. A. & SMYDA, M. A. 2012. High-Antioxidant Potatoes: Acute in Vivo Antioxidant Source and Hypotensive Agent in Humans after Supplementation to Hypertensive Subjects. *J Agric Food Chem*.

VINSON, J. A. & HOWARD, T. B. 1996. Inhibition of protein glycation and advanced glycation end products by ascorbic acid and other vitamins and nutrients. *Journal of Nutritional Biochemistry,* 7**,** 659-663.

VIRTAMO, J., PIETINEN, P., HUTTUNEN, J. K., KORHONEN, P., MALILA, N., VIRTANEN, M. J., ALBANES, D., TAYLOR, P. R. & ALBERT, P. 2003. Incidence of cancer and mortality following alpha-tocopherol and beta-carotene supplementation: a postintervention follow-up. *JAMA,* 290**,** 476-85.

VITAGLIONE, P., DONNARUMMA, G., NAPOLITANO, A., GALVANO, F., GALLO, A., SCALFI, L. & FOGLIANO, V. 2007. Protocatechuic Acid Is the Major Human Metabolite of Cyanidin-Glucosides. *The Journal of Nutrition,* 137**,** 2043-2048.

VITEK, M. P., BHATTACHARYA, K., GLENDENING, J. M., STOPA, E., VLASSARA, H., BUCALA, R., MANOGUE, K. & CERAMI, A. 1994. Advanced glycation end products contribute to amyloidosis in Alzheimer disease. *Proc Natl Acad Sci U S A,* 91**,** 4766-70.

VLASSARA, H. 1996. Advanced glycation end-products and atherosclerosis. *Ann Med,* 28**,** 419-26.

VLASSARA, H., LI, Y. M., IMANI, F., WOJCIECHOWICZ, D., YANG, Z., LIU, F. T. & CERAMI, A. 1995. Identification of galectin-3 as a high-affinity binding protein for advanced glycation end products (AGE): a new member of the AGE-receptor complex. *Mol Med,* 1**,** 634-46.

VLASSARA, H. & PALACE, M. R. 2002. Diabetes and advanced glycation endproducts. *J Intern Med,* 251**,** 87-101.

VLASSOPOULOS, A., LEAN, M. & COMBET, E. 2013a. Influence of smoking and diet on glycated haemoglobin and 'pre-diabetes' categorisation: a cross-sectional analysis. *BMC Public Health,* 13**,** 1013.

VLASSOPOULOS, A., LEAN, M. E. & COMBET, E. 2013b. Role of oxidative stress in physiological albumin glycation: a neglected interaction. *Free Radic Biol Med,* 60**,** 318-24.

VOIGT, M., JORCH, G., BRIESE, V., KWOLL, G., BORCHARDT, U. & STRAUBE, S. 2011. The combined effect of maternal body mass index and smoking status on perinatal outcomes - an analysis of the german perinatal survey. *Z Geburtshilfe Neonatol,* 215**,** 23-8.

VOS, P. A. J. M., DEGROOT, J., HUISMAN, A. M., OOSTVEEN, J. C. M., MARIJNISSEN, A. C. A., BIJLSMA, J. W. J., VAN EL, B., ZUURMOND, A. M. & LAFEBER, F. P. J. G. 2010. Skin and urine pentosidine weakly correlate with joint damage in a cohort of patients with early signs of osteoarthritis (CHECK). *Osteoarthritis and Cartilage,* 18**,** 1329-1336.

VOZIYAN, P. A., KHALIFAH, R. G., THIBAUDEAU, C., YILDIZ, A., JACOB, J., SERIANNI, A. S. & HUDSON, B. G. 2003. Modification of proteins in vitro by physiological levels of glucose: pyridoxamine inhibits conversion of Amadori intermediate to advanced glycation end-products through binding of redox metal ions. *J Biol Chem,* 278**,** 46616-24.

WANG, H. Y., QIAN, H. & YAO, W. R. 2011. Melanoidins produced by the Mail lard reaction: Structure and biological activity. *Food Chemistry,* 128**,** 573-584.

WAUTIER, J.-L. & SCHMIDT, A. M. 2004. Protein Glycation: A Firm Link to Endothelial Cell Dysfunction. *Circulation Research,* 95**,** 233-238.

WEDICK, N. M., PAN, A., CASSIDY, A., RIMM, E. B., SAMPSON, L., ROSNER, B., WILLETT, W., HU, F. B., SUN, Q. & VAN DAM, R. M. 2012. Dietary flavonoid intakes and risk of type 2 diabetes in US men and women. *Am J Clin Nutr,* 95**,** 925-33.

WEISS, J. L., MALONE, F. D., EMIG, D., BALL, R. H., NYBERG, D. A., COMSTOCK, C. H., SAADE, G., EDDLEMAN, K., CARTER, S. M., CRAIGO, S. D., CARR, S. R. & D'ALTON, M. E. 2004. Obesity, obstetric complications and cesarean delivery rate–a population-based screening study. *American Journal of Obstetrics and Gynecology,* 190**,** 1091-1097.

WEYKAMP, C. W., PENDERS, T. J., BAADENHUIJSEN, H., MUSKIET, F. A., MARTINA, W. & VAN DER SLIK, W. 1995. Vitamin C and glycohemoglobin. *Clin Chem,* 41**,** 713-6.

WHO 2010. United Nations: World Population Prospects: The 2008 Revision, Volume 1: Comprehensive Tables and United Nations, World Population Prospects: The 2008 Revision, Highlights. *Population and Development Review,* 36**,** 854-855.

WHO 2011. *Global status report on noncommunicable diseases 2010,* Geneva, Switzerland, World Health Organization.

WHO 2014. World Population Prospects, the 2012 Revision.

WILDMAN, R. P., MUNTNER, P., REYNOLDS, K., MCGINN, A. P., RAJPATHAK, S., WYLIE-ROSETT, J. & SOWERS, M. R. 2008. The obese without cardiometabolic risk factor clustering and the normal weight with cardiometabolic risk factor clustering: prevalence and correlates of 2 phenotypes among the US population (NHANES 1999-2004). *Arch Intern Med,* 168**,** 1617-24.

WILLI, C., BODENMANN, P., GHALI, W. A., FARIS, P. D. & CORNUZ, J. 2007. Active smoking and the risk of type 2 diabetes: a systematic review and meta-analysis. *JAMA,* 298**,** 2654-64.

WILLIAMSON, G. & MANACH, C. 2005. Bioavailability and bioefficacy of polyphenols in humans. II. Review of 93 intervention studies. *American Journal of Clinical Nutrition,* 81**,** 243s-255s.

WING, R. R., LANG, W., WADDEN, T. A., SAFFORD, M., KNOWLER, W. C., BERTONI, A. G., HILL, J. O., BRANCATI, F. L., PETERS, A., WAGENKNECHT, L. & GROUP, T. L. A. R. 2011. Benefits of Modest Weight Loss in Improving Cardiovascular Risk Factors in Overweight and Obese Individuals With Type 2 Diabetes. *Diabetes Care,* 34**,** 1481-1486.

WISEMAN, M. 2008. The second World Cancer Research Fund/American Institute for Cancer Research expert report. Food, nutrition, physical activity, and the prevention of cancer: a global perspective. *Proc Nutr Soc,* 67**,** 253-6.

WITKOSARSAT, V., FRIEDLANDER, M., CAPEILLEREBLANDIN, C., NGUYENKHOA, T., NGUYEN, N. T., ZINGRAFF, J., JUNGERS, P. & DESCAMPSLATSCHA, B. 1996. Advanced oxidation protein products as a novel marker of oxidative stress in uremia. *Kidney International,* 49**,** 1304-1313.

WOLFF, M. S., COLLMAN, G. W., BARRETT, J. C. & HUFF, J. 1996. Breast cancer and environmental risk factors: epidemiological and experimental findings. *Annu Rev Pharmacol Toxicol,* 36**,** 573-96.

WOLFF, S. P. & DEAN, R. T. 1987. Glucose autoxidation and protein modification. The potential role of 'autoxidative glycosylation' in diabetes. *Biochem J,* 245**,** 243-50.

WOLFF, S. P., JIANG, Z. Y. & HUNT, J. V. 1991. Protein glycation and oxidative stress in diabetes mellitus and ageing. *Free Radic Biol Med,* 10**,** 339-52.

WOOD, I. S., DE HEREDIA, F. P., WANG, B. & TRAYHURN, P. 2009. Cellular hypoxia and adipose tissue dysfunction in obesity. *Proceedings of the Nutrition Society,* 68**,** 370-377.

WU, C. H. & YEN, G. C. 2005. Inhibitory effect of naturally occurring flavonoids on the formation of advanced glycation endproducts. *Journal of Agricultural and Food Chemistry,* 53**,** 3167-3173.

WU, J. W., HSIEH, C. L., WANG, H. Y. & CHEN, H. Y. 2009. Inhibitory effects of guava (Psidium guajava L.) leaf extracts and its active compounds on the glycation process of protein. *Food Chemistry,* 113**,** 78-84.

WYNDER, E. L., ROSE, D. P. & COHEN, L. A. 1994. Nutrition and prostate cancer: a proposal for dietary intervention. *Nutr Cancer,* 22**,** 1-10.

XI, M., HAI, C., TANG, H., CHEN, M., FANG, K. & LIANG, X. 2008. Antioxidant and antiglycation properties of total saponins extracted from traditional Chinese medicine used to treat diabetes mellitus. *Phytother Res,* 22**,** 228-37.

XIAO, J. B. & KAI, G. Y. 2012. A Review of Dietary Polyphenol-Plasma Protein Interactions: Characterization, Influence on the Bioactivity, and Structure-Affinity Relationship. *Critical Reviews in Food Science and Nutrition,* 52**,** 85-101.

XIE, Y. & CHEN, X. 2013. Structures required of polyphenols for inhibiting advanced glycation end products formation. *Curr Drug Metab,* 14**,** 414-31.

YAN, S. F., RAMASAMY, R., NAKA, Y. & SCHMIDT, A. M. 2003. Glycation, inflammation, and RAGE - A scaffold for the macrovascular complications of diabetes and beyond. *Circulation Research,* 93**,** 1159-1169.

YAN, S. F., RAMASAMY, R. & SCHMIDT, A. M. 2010. The RAGE axis: a fundamental mechanism signaling danger to the vulnerable vasculature. *Circ Res,* 106**,** 842-53.

YU, Y., HANSSEN, K. F., KALYANARAMAN, V., CHIRINDEL, A., JENKINS, A. J., NANKERVIS, A. J., TORJESEN, P. A., SCHOLZ, H., HENRIKSEN, T., LORENTZEN, B., GARG, S. K., MENARD, M. K., HAMMAD, S. M., SCARDO, J. A., STANLEY, J. R., WU, M., BASU, A., ASTON, C. E. & LYONS, T. J. 2012. Reduced soluble receptor for advanced glycation end-products (sRAGE) scavenger capacity precedes pre-eclampsia in Type 1 diabetes. *BJOG,* 119**,** 1512-20.

YUDKIN, J. S., STEHOUWER, C. D. A., EMEIS, J. J. & COPPACK, S. W. 1999. C-Reactive Protein in Healthy Subjects: Associations With Obesity, Insulin Resistance, and Endothelial Dysfunction: A Potential Role for Cytokines Originating From Adipose Tissue? *Arteriosclerosis, Thrombosis, and Vascular Biology,* 19**,** 972-978.

YUN, T. K. 2003. Experimental and epidemiological evidence on non-organ specific cancer preventive effect of Korean ginseng and identification of active compounds. *Mutat Res,* 523-524**,** 63-74.

ZHANG, Q. B., AMES, J. M., SMITH, R. D., BAYNES, J. W. & METZ, T. O. 2009. A Perspective on the Maillard Reaction and the Analysis of Protein Glycation by Mass Spectrometry: Probing the Pathogenesis of Chronic Disease. *Journal of Proteome Research,* 8**,** 754-769.

ZHANG, X., CHEN, F. & WANG, M. 2014. Antioxidant and Antiglycation Activity of Selected Dietary Polyphenols in a Cookie Model. *Journal of Agricultural and Food Chemistry,* 62**,** 1643-1648.

ZIEMAN, S. J., MELENOVSKY, V., CLATTENBURG, L., CORRETTI, M. C., CAPRIOTTI, A., GERSTENBLITH, G. & KASS, D. A. 2007. Advanced glycation endproduct crosslink breaker (alagebrium) improves endothelial function in patients with isolated systolic hypertension. *J Hypertens,* 25**,** 577-83.

Appendix