Non-dietary determinants and correlates of plasma concentrations of lutein and zeaxanthin in an Irish population

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Declaration

No element of the work described in this Thesis or the Thesis itself, except where otherwise acknowledged, has been previously submitted for a degree at this or any other institution. The work described in this Thesis has been performed entirely by the author.

Signature _____ Date _____

Dedication

I dedicate this thesis to the loving memory of my dad, Terry, a man who always focused on what he needed more than what he wanted. Until it came to his daughters, then he wanted to give us the moon and more.

"I don't need very much now," said the boy.

"just a quiet place to sit and rest.

I am very tired."

"Well," said the tree, straightening

herself up as much as she could,

"well, an old stump is good for sitting and resting

Come, Boy, sit down. Sit down and rest."

And the boy did.

And the tree was happy.

"The Giving Tree"

by Shel Silverstein

Acknowledgments

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List of Abbreviations (in alphabetical order)

Acceptable daily intake	ADI
Age-related Eye Disease Study	AREDS
Age-related macular degeneration	AMD
Alzheimer's disease	AD
Anti-vascular endothelial growth factor	anti-VEGF
Autofluorescence	AF
Beaver Dam Eye Study	BDES
Blue Mountain Eye Study	BMES
Body mass index	BMI
Butylated Hydroxytoluene	BHT
Cardiovascular disease	CVD
Carotenoids and Age-related Dementia study	CARDS
Carotenoids in Age-related Eye Disease Study	CAREDS
Central Retinal Enrichment Supplementation Trials	CREST
Choroidal neovascularization	CNV
Computer-aided personal interview	CAPI
Confocal scanning laser ophthalmoscope	SLO
Coronary heart disease	CHD
Customized heterochromatic flicker photometry	cHFP
Diabetic retinopathy	DR
Ethylene diamine tetra-acetic acid	EDTA
European Prospective Investigation into Cancer and Nutrition	EPIC
European Eye study	EUREYE
Eye Disease Case Control Study	EDCCS
Food frequency questionnaire	FFQ

Gap junction communication	GJC
Geographic atrophy	GA
Grams	g
Height of baseline noise	h
Height of peak	H
Heterochromatic flicker photometry	HFP
High density lipoproteins	HDL
High performance liquid chromatography	HPLC
Internal standard	IS
Kilograms	kg
Limit of detection	LOD
Limit of quantification	LOQ
Low density lipoproteins	LDL
Lutein	L
Mass spectrometry	MS
Macular Pigment	MP
Macular Pigment Research Group	MPRG
Metre	m
Methyl tert-butyl ether	MTBE
Meso-zeaxanthin	MZ
Meso-zeaxanthin Ocular Supplementation Trials	MOST
Micrograms	µg
Milligrams	mg
Muenster Ageing and Retina	MARS
National Health and Nutrition Examination Eye Survey	NHANES
National Institute of Standards and Technology	NIST
Nanometre	nm
Number of participants	n

Nurses' Health Study	NHS
Nutrition Research Centre Ireland	NRCI
Organisation for Economic Co-operation and Development	OECD
Peak area	PA
Pathologies Oculaires Liees a I'Age Study	POLA
Polyunsaturated fatty acids	PUFAs
RANdom SAMpling design for Ireland	RANSAM
Reactive oxygen species	ROS
Relative standard deviation	RSD
Republic of Ireland	ROI
Response factor	RF
Retinal pigment epithelium	RPE
Self-completion questionnaire	SCQ
Signal to noise ratio	S/N
Standard deviation	SD
Standard operating procedure	SOP
Standard reference material	SRM
Total cholesterol	TC
The Irish LongituDinal Study on Aging	TILDA
Ultraviolet	UV
Ultraviolet-visible	UV-Vis
United States Department of Agriculture	USDA
World Health Organisation	WHO
Zeaxanthin	Z

Abstract

The macula is a specialised area of the retina that mediates central and colour vision. Three dietary carotenoids, lutein (L), zeaxanthin (Z) and *meso*-zeaxanthin (MZ), accumulate in the macula, where they are collectively referred to as macular pigment (MP). Age-related macular degeneration (AMD) is a disease of the macula that, in its advanced stage, results in a loss of central vision. The light-filtering and antioxidant properties of MP render this pigment important for optimising visual function and protecting against AMD. The current investigation (conducted as part of The Irish Longitudinal Study on Ageing [TILDA]) is the first study to report plasma concentrations of L and Z in a large representative sample of the Irish population. TILDA is a comprehensive study on the health, economic and social status of over 8,000 Irish adults aged 50 years and over. The main focus of this study was to quantify and assess plasma concentrations of L and Z from TILDA participants, and investigate their association with the prevalence of AMD, and non-dietary determinants of these carotenoids. Firstly, the findings from this study indicated that plasma concentrations of L and Z are lower in association with indicators of a poor lifestyle (high BMI, tobacco use, and less physical exercise) and lower education, indicating that modifying lifestyle in a positive way is likely to be reflected in higher concentrations of plasma carotenoids with consequential health benefits. The second part of this study indicated that plasma concentrations of L and Z were significantly higher in association with grading-confirmed presence of AMD and awareness (self-report) of AMD, which is likely due to greater supplement use in these participants. This research will contribute to the existing scientific literature, and will hopefully guide healthcare and medical practice regarding the importance of macular carotenoids for eye health and general wellbeing.

Chapter 1: Literature review

1.1 Methods of literature search

The following is a list of keywords, and combination of words, which was used to perform the literature search using PubMed, Science Direct and Google Scholar: *free radical; ageing; oxidative stress; antioxidants; carotenoids; lutein; zeaxanthin; meso-zeaxanthin; macular pigment; retina; macula; risk factors; determinants; correlates; age-related macular degeneration; epidemiology*.

1.2 Thesis outline

This chapter highlights the appropriate background literature on the ageing process and associated health problems such as age-related macular degeneration (AMD), with an emphasis on the location, function and source of the macular carotenoids, lutein (L), *meso*-zeaxanthin (MZ), and zeaxanthin (Z).

Chapter 2 focuses on the design, sampling and methodology used in The Irish Longitudinal Study on Ageing (TILDA). This chapter also discusses the method development for the quantification of L and Z from human plasma by high performance liquid chromatography (HPLC), which was achieved to efficiently and accurately quantify over 5,000 plasma samples.

Chapter three presents my first report investigating non-dietary determinants and correlates of plasma concentrations of L and Z in TILDA participants. Chapter four

presents my second report investigating the relationships between plasma concentrations of L and Z and grading-confirmed AMD in TILDA participants. Chapter five summarises the main findings, implications of these findings and future considerations. Of note, references are located at the end of each chapter except for Chapter three and four, which are combined at the end of Chapter four.

1.3 Introduction

Research designed to investigate the health status of older adults is one of the most important global challenges for society. In the early 20th century, age expectancy did not exceed past age 50 (years), whereas, the average life expectancy at birth has increased to 71.4 years as of 2015.[1] The world's population is rapidly ageing due to improvements made in global health. By 2050, there will be more elderly people than children worldwide.[2] Chronic diseases such as AMD,[3] Alzheimer's disease (AD),[4] cardiovascular disease (CVD),[5] and many other illnesses are becoming more prevalent among older adults. This leads to a myriad of questions: will good health and wellbeing coincide with ageing? Or will ageing be defined by illness, dependency and disabilities? What implications will ageing have on healthcare and associated costs? What effect will ageing have on social dependence of these individuals and their families?

Like most countries in the world, Ireland is experiencing a dramatic rise in those aged 65 years and over.[6] We, as a nation, are faced with the heavy task of ensuring that the quality of life is able to keep up with the challenging relationship between increasing age and wellbeing. Global efforts are needed to combine reliable databases

and comprehensive research that will provide a better understanding of the ageing process, which in turn will strengthen the potential treatment and management of age-related diseases.

Observational and interventional studies, which have been conducted worldwide to enhance the knowledge about disease prevalence, incidence, and the outcome of treatments, are required to successfully win the battle against diseases associated with ageing. Indeed, and specific to my research, given the potential health and functional benefits of carotenoids in humans (discussed fully below), it is important to study predictors (both modifiable and non-modifiable) of these nutrients in an ageing population. In order to achieve this, my research availed of the unique opportunity that TILDA offered to study circulating plasma concentrations of the macular carotenoids, L and Z, in association with demographic, lifestyle, and health variables in older Irish adults.

1.4 Free radicals, antioxidants and ageing

1.4.1 Free radicals

Free radicals are atoms or molecules with one or more unpaired electrons.[7] As a result, these highly reactive radicals "steal" electrons from the nearest molecule, which sets off a chain reaction when the nearest molecule becomes unstable and attempts to become stable by "stealing" other electrons. Reactive oxygen species (ROS) are free radicals derived from oxygen and when generated at moderate levels are important for physiological functions.[8] While oxygen is required to live, high concentrations can be

toxic and generate dangerous by-products. As part of normal aerobic metabolism, the overproduction of ROS and ROS precursors such as singlet oxygen, superoxide, hydrogen peroxide, hydroxyl radical, and hydrogen ion cause damage to proteins, DNA and cellular lipids. In our environment, rusting metal and rotting fruit are examples of free radical activity. Inside our bodies, free radicals can cause cumulative oxidative damage, resulting in ageing and chronic illness.[8]

1.4.2 Antioxidants

Antioxidants are defence molecules, which can safely interact with free radicals and terminate the chain reaction or quench singlet oxygen before cellular damage occurs. Animals can either produce antioxidants in the body (e.g. glutathione and catalase) or obtain antioxidants through their diet to interact and neutralise free radicals (Figure 1.1). Dietary antioxidants such as ascorbic acid (vitamin C), tocopherols (vitamin E) and carotenoids act as a defence against oxidative stress by scavenging free radicals.

Tocopherols are fat-soluble antioxidants (α-, β-, γ-, δ-tocopherol, and α-, β-, γ-, δ-tocotrienol) that protect fatty acids from lipid peroxidation; ascorbic acid is a water-soluble antioxidant, which quenches ROS and also regenerates tocopherols; carotenoids are plant pigments that quench singlet oxygen (ROS) and other excited molecules.[8] Antioxidants such as carotenoids have been studied for their ability to reduce the risk of several chronic health disorders, including certain cancers and eye diseases.[9] For this reason, there is considerable interest in the biological and chemical function of carotenoids, which is discussed in this chapter.

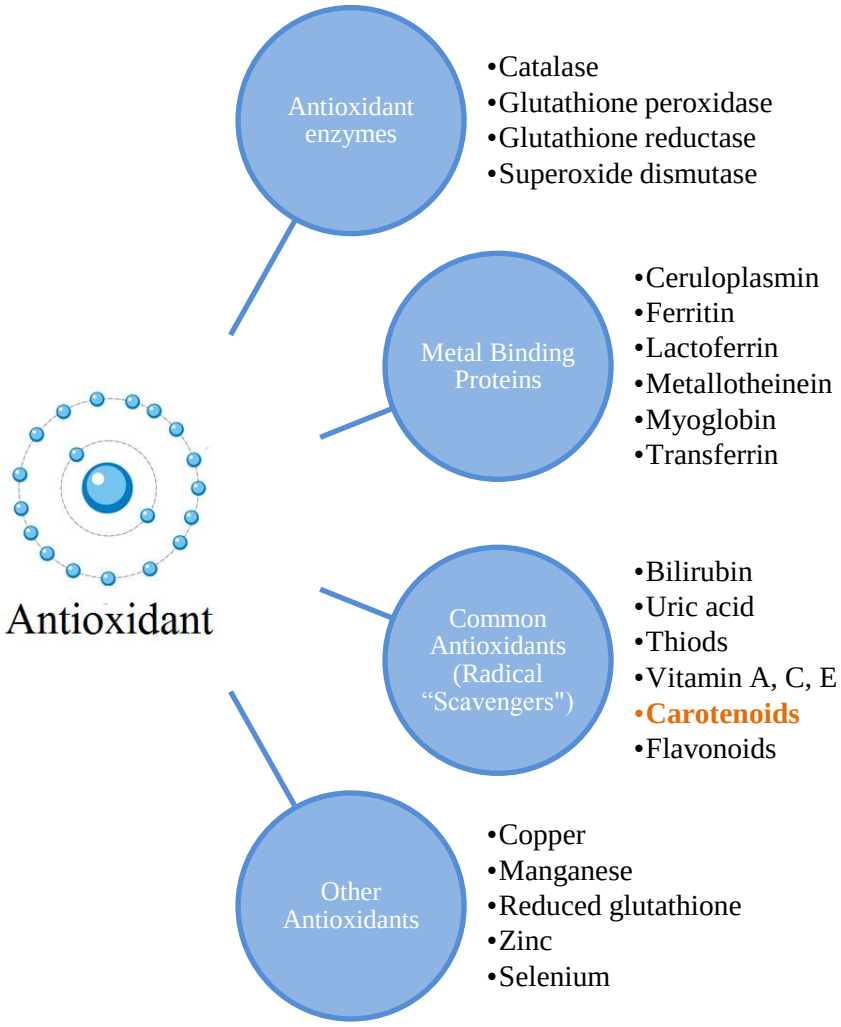

Figure 1.1: Examples of natural antioxidants.

1.4.3 Oxidative process

Oxidative stress occurs when there is an imbalance between the pro-oxidants (chemicals that generate ROS) and antioxidant status, thus leading to oxidative damage to cells and tissue. The accumulation of cellular damage over time has been recognised as a contributing factor to ageing.[10, 11] Oxidative damage is associated with age-related

diseases such as cardiovascular disease, and neurodegenerative disease (Parkinson's disease and AD).[11]

1.4.4 Phenomenon of ageing

As we age, vital parts of our cells, tissues, and organs lose their ability to function normally (i.e. damage to cellular components and ultimately cell death). Denham Harman hypothesised that damage caused by free radicals, produced as part of normal metabolic processes, was responsible for the changes that results from ageing.[10] Consequently, the free radical theory of ageing was proposed along with many other theories on the phenomenon of ageing such as molecular cross-linking,[12] changes in immunologic function,[13] and senescence genes in the DNA.[14]

1.4.5 Healthy ageing

During the 20th century, increased life expectancy was successfully achieved (World Health Organization [WHO] reports life expectancy at birth exceeds 83 years in Japan). However, we must consider if an increased life expectancy will mean spending more years in poor health. Life expectancy has increased due to improvements in technology and medicine, however we are now facing the burden of age-associated diseases that had been previously masked by shorter life expectancies. Healthy ageing involves interactions between our genes and the environment (e.g. lifestyle) that leads to optimal health. If lifestyle is poor and the investment in body maintenance is not optimal,

negative ageing occurs. However, increasing our understanding of the ageing process and implementing appropriate interventions could positively enhance healthy ageing.

Nutrition, the intake of food, influences all processes (i.e. maintenance, growth, and reproduction for cells) essential for the maintenance of life and health.[15] There is growing knowledge of how specific nutrients can account for significant health and performance benefits.[16] For the most part, we have focused on promoting adequate nutrition in terms of early growth and development during pregnancy and for infants. However, nutritional guidance may be less stringent as adults, resulting in reduced wellness, compromised performance, and a significantly increased risk for the development of age-related disease.

1.5 Carotenoids

Carotenoids are a class of over 750 naturally occurring pigments synthesised by plants and algae (found in their chloroplasts and chromoplasts), as well as bacteria, yeast and fungi.[17] Most carotenoids are composed of eight branched C_5 isoprenoid units attached in a head-to-tail pattern (two C_{20} units), where a head-to-head linkage at the centre of the molecule results in a symmetric molecule (Figure 1.2).[18] Most carotenoids are hydrophobic and lipophilic and are typically located inside the cell membrane.

Carotenoids absorb light between 400-550 nanometres (nm) and range from pale yellow to vibrant red. Their colour is determined by the chromophore length (i.e. absorption spectrum). These organic pigments are the source of the rich, bright colours

we see in flowers (e.g. marigold), fruits (e.g. tomatoes), vegetables (e.g. corn), and animals (e.g. salmon flesh and flamingos' feathers).

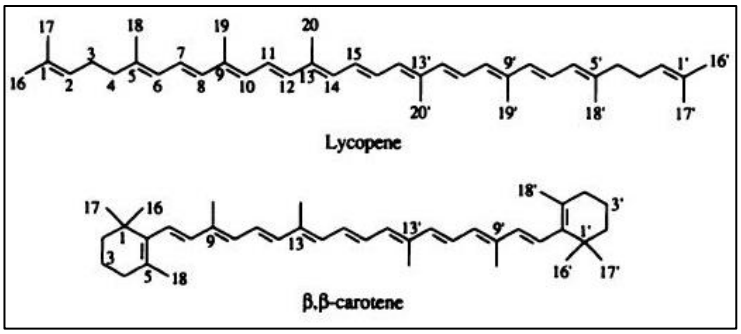

Figure 1.2: Basic structure and number scheme of lycopene and β,β-carotene.[2]

1.5.1 Class of carotenoids

Carotenoids are split into two classes: carotenes (purely hydrocarbons and contain no oxygen) and xanthophylls (oxygen derivatives and more polar than carotenes). Carotenes are located within the inner part of the lipid bi-layer, while xanthophylls are positioned perpendicular to the membrane surface.[19] Animals and humans are unable to synthesise carotenoids and as a result, the level of carotenoid is dependent on dietary intake of these nutrients. Approximately fifty carotenoids are constituents in the human diet, while *circa* eighteen are present in human blood.[20-22] The most abundant carotenoids in foods include beta-carotene, alpha-carotene, lycopene, L, Z and beta-cryptoxanthin (Figure 1.3).

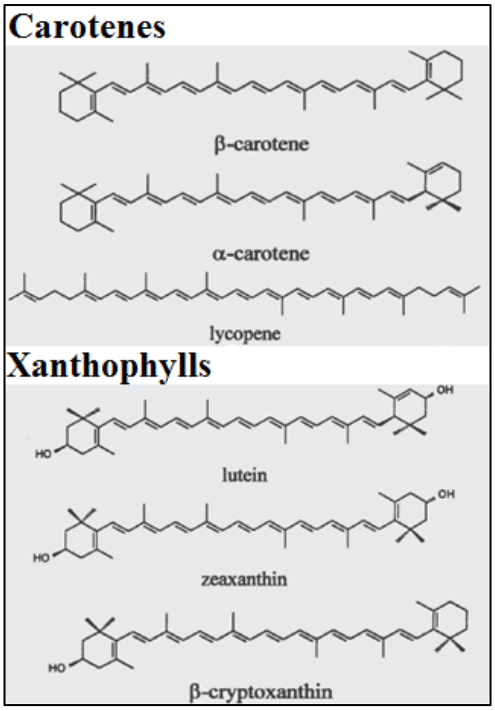

Figure 1.3: Chemical structure of carotenoids found in plasma.[23]

1.5.2 Antioxidant properties of carotenoids

Scientific evidence has suggested that carotenoids protect cells from the damage caused by free radicals and reduce the risk of developing several chronic health disorders.[24, 25] Carotenoids can also quench singlet oxygen by transferring the excess energy from singlet oxygen to the carotenoids' electron-rich structure and dispersing the extra energy as heat. The conjugated double bonds, found in all carotenoids, are responsible for their antioxidant activity.[26, 27] Some carotenoids (e.g. beta-carotene) have additional health benefits such as the ability to be converted to Vitamin A, which helps promote a healthy immune system and cell growth. Vitamin A is also the precursor of the rhodopsin chromophore retinal, which is critical for night vision.

1.5.3 Photochemical properties of carotenoids

As previously mentioned, carotenoids are vibrant yellow, orange or red as their main absorption bands lie between 400 and 550 nm (visible region) (Figure 1.4). This is attributed to a strong transition from the ground state (S_0) to the second singlet excited state (S_2). The energy levels of S_2 and first excited state (S_1) (demotion of S_2 via internal conversion) lie close to that of chlorophyll in plants. The singlet energy transfers from the excited carotenoids generate the excited singlet state S_1 of chlorophyll, which is active in photosynthesis.[28] In summary, as a result of their conjugated polyene chain, carotenoids are intensely coloured and exhibit excellent light-harvesting and photo-protective action.

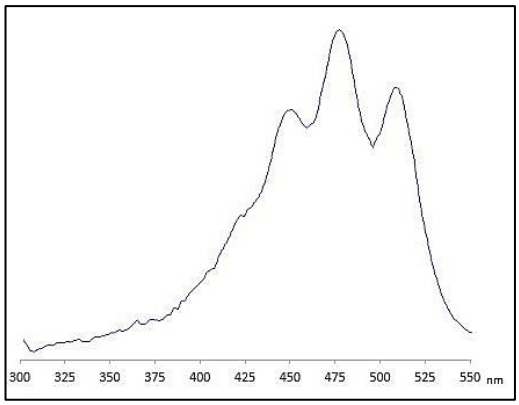

Figure 1.4: Absorbance spectrum of lycopene.

1.5.4 Other properties of carotenoids

In addition to the aforementioned properties, the ability of carotenoids to stimulate gap junction communication (GJC) has been discussed as a possible biochemical mechanism for protection against cancers.[29-31] Gap junctions (specialised intercellular

channels) permit cell-cell transfer of electric currents, small molecules and ions between neighbouring cells. A gap junction is composed of two connexions (6 proteins each), which are responsible for the transmission of intercellular communication. The loss of gap junction communication has been deemed a hallmark of carcinogenesis (formation of cancer).[32] Carotenoids have been shown to simulate gap junction communication via increased levels of connexion 43[33] and inhibit the proliferation of cancer cells.[34] In addition, the expression and functions of neuronal gap junction (connexion 36, 45, 57) may play a role in the retina of mice.[35, 36] A full understanding of the mode in which carotenoids affect cell proliferation is still unclear.

Because of the relation between ROS and inflammation, carotenoids and their metabolites may also play a part in inhibiting inflammatory process by interacting with cellular signalling cascades and reducing ROS.[37] During this process, cells of the immune system (macrophages and leucocytes) are recruited to the site of damage and produce mediators such as cytokines and chemokines, which activate different signal transduction cascades and transcription factors.[38] Carotenoids have been found to positivity modulate markers of inflammation and oxidative stress by influencing transcription factors (e.g. NF- κ B or Nrf2 pathway).[37]

1.5.5 *Cis* and *trans* isomers

The Latin terms "*cis*" and "*trans*" are used in organic chemistry to describe stereoisomers. Stereoisomers are molecules with the same molecular formula and same sequence of bonded atoms, but a different arrangement of the atoms in space. *Cis*

indicates that the functional groups are on the same side of the carbon chain, while *trans* indicates that the functional groups are on the opposite side of the carbon chain (Figure 1.5). *Cis* isomers (restricted rotation in the molecule) can occur as a result of light, heat and other conditions in nature. Although carotenoids exist in plants as the all-*trans* (or all-E) form, they are susceptible to geometrical isomerisation and can exist as *cis* isomers.

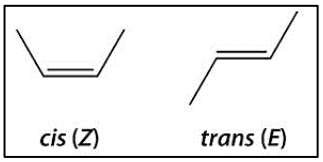

Figure 1.5: *Cis*- and *trans*- configurations.

The occurrence of *cis*-carotenoids (Figure 1.6) in plasma could be explained by the fact there is a small mixture of isomers in foods as a result of thermal processing.[39] *Cis*- and *trans*- have different chemical and physical properties. Therefore, it is essential to separate and quantify *cis*-isomers from their *trans* form as very little is known about the biological significance of carotenoid isomerisation in human health.[40]

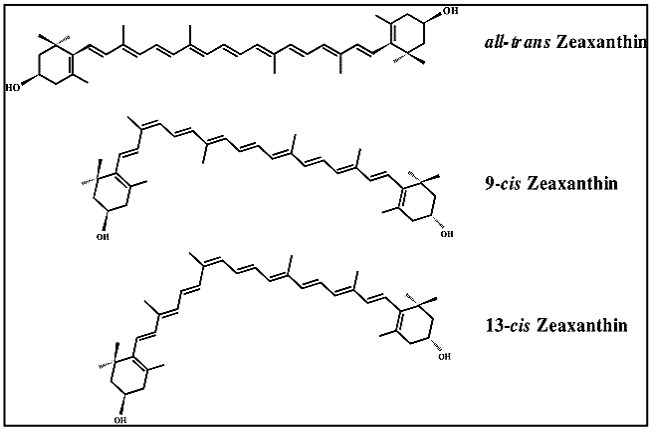

Figure 1.6: Common geometric isomers of zeaxanthin.[41]

1.5.6 Bioavailability of carotenoids

The chemical and physical properties of carotenoids are also influenced by interactions with other molecules such as lipids and proteins.[28] As with many nutrients, the biological activity of carotenoids depends on their bioavailability and uptake in the gastrointestinal tract. Dietary factors and non-dietary factors can affect the bioavailability of carotenoids. The mnemonic "SLAMENGHI" describes these factors: species of carotenoids, linkages at molecular level, amount of carotenoid, matrix, effectors, nutrient status, genetics, host related factors and interactions among these variables.[42] The type of food matrix and food processing, particularly heat treatment and consumption with oils, have the potential to improve the bioavailability of carotenoids from foods. Absorption requires the digestion of the food matrix, the release of the carotenoids, formation of lipid micelles in the small intestine (Figure 1.7), uptake of carotenoids by intestinal mucosal cells, and finally transport of carotenoids to the bloodstream as illustrated in Figure 1.8.[43]

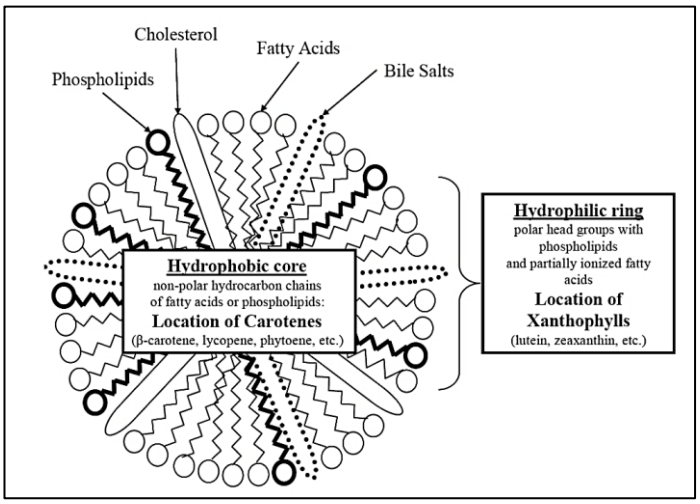

Figure 1.7: Location of carotenoids in lipid micelles.[44]

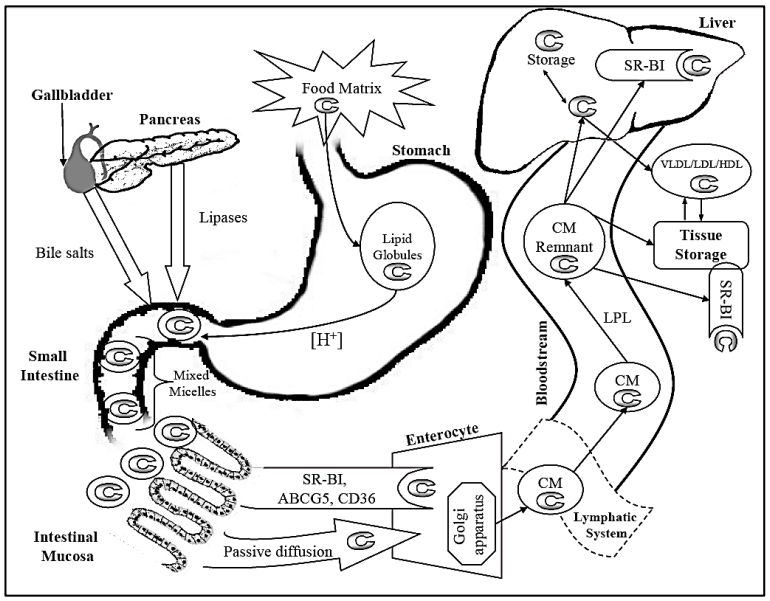

Figure 1.8: Pathway involved in the absorption, transport and uptake of carotenoids.[44]

Once in the blood stream, carotenoids are taken up by the liver and transported in plasma exclusively by lipoproteins to various target tissues in the body (e.g. the retina and brain). The carotenoid's physical properties determine their distribution among the lipoprotein classes. Hydrocarbon carotenoids such as beta-carotene, alpha-carotene and lycopene are transported on low density lipoproteins (LDL), whereas xanthophylls (L and Z) have an affinity towards high density lipoproteins (HDL).[45] For example, tissues (e.g. adrenal glands) high in LDL receptors would be high in carotenes,[46] while the specific binding or affinity of the xanthophylls to HDL are more efficiently delivered to the central nervous system and retina.[47] This has led to the suggestion that an individual's lipoprotein profile could influence the transport and delivery of carotenoid to tissues.[48]

1.6 Macular pigment (MP)

The xanthophylls, L, MZ, and Z, collectively known as macular pigment (MP), account for the yellow coloration at the macula (see section 1.6.1).[49, 50] The hydroxyl group at each end of the L, MZ and Z (also referred to as macular carotenoids) differentiates the molecules from other carotenoids.[51] The chemical structure may determine the why and where, while the biological function could decide when and how the body may use it. Even their orientation and location of single double bond has a huge difference on the distribution in eye and impact on their function within the eye (Figure 1.9).[52] L contains both a β-ring and an ε-ring and so its polyene chain is slightly shorter than Z and MZ, which both contain two β-ring end groups. The macular carotenoids also differ in terms of the spatial orientation of their hydroxyl group. L has three chiral centres with the configuration (3R,3′R,6′R); Z has two chiral centres configured as (3R,3′R), while MZ, also with two chiral centres has the configuration (3R,3′S).[50]

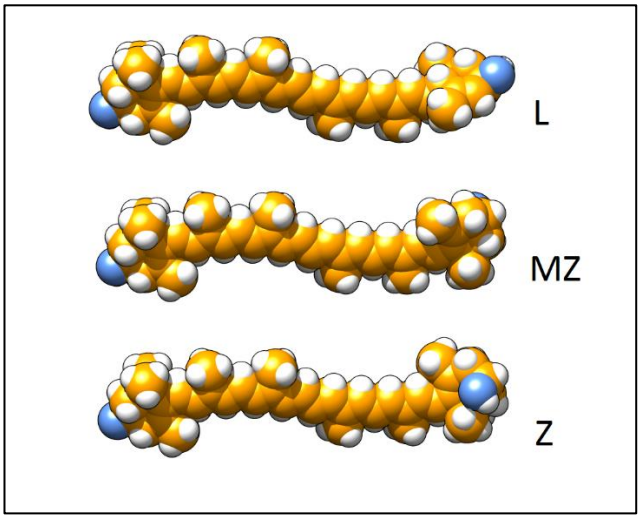

Figure 1.9: Chemical structure of lutein (L), meso-zeaxanthin (MZ) and zeaxanthin (Z). Image courtesy of Dr Alfonso Prado-Cabrero, Waterford, Ireland.

The discovery of MP dates back as early as the 17th century (Figure 1.10). Limited by the lack of specialised tools and technology at that time, investigation of MP relied heavily on the methodical and precise dissection of the eye. MP has generated interest in recent years because of its possible protective role for eye diseases such as AMD (Figure 1.10).

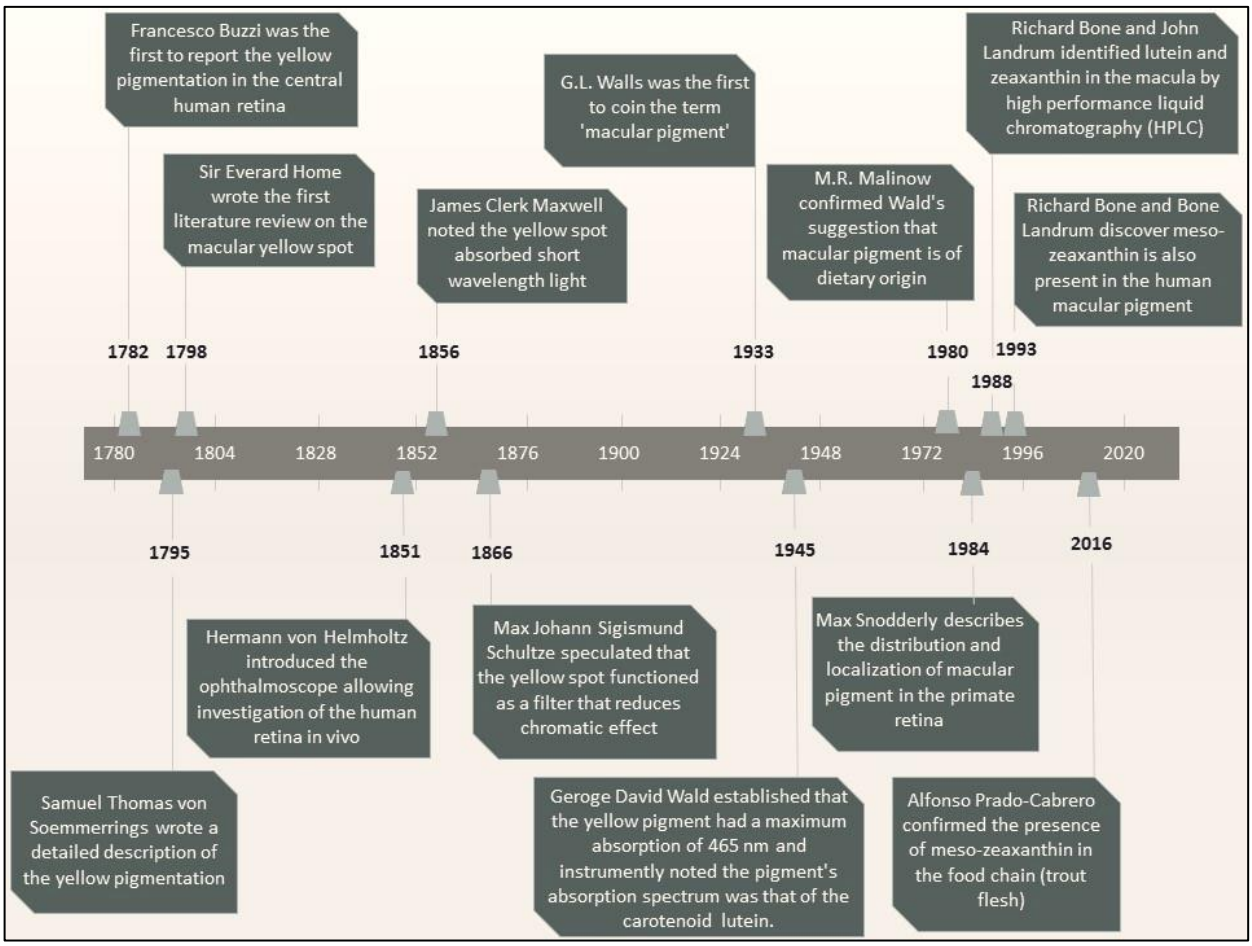

Figure 1.10: History of macular pigment (1782-2016).

1.6.1 Location and distribution of MP

The macula or *macula lutea* (Latin for "yellow spot") is a small extra-sensitive spot near the centre of the retina that gives sharp, central vision (Figure 1.11). The retina is a layered structure and contains two types of photoreceptors: rods and cones. The rods provide black and white vision and are much more sensitive in terms of absolute threshold for light detection than cones. There are more rods than cones; however, cones are much more concentrated in the macula. Cones are responsible for colour and high resolution vision, and are concentrated at the macula where they correspond with the distribution of MP.

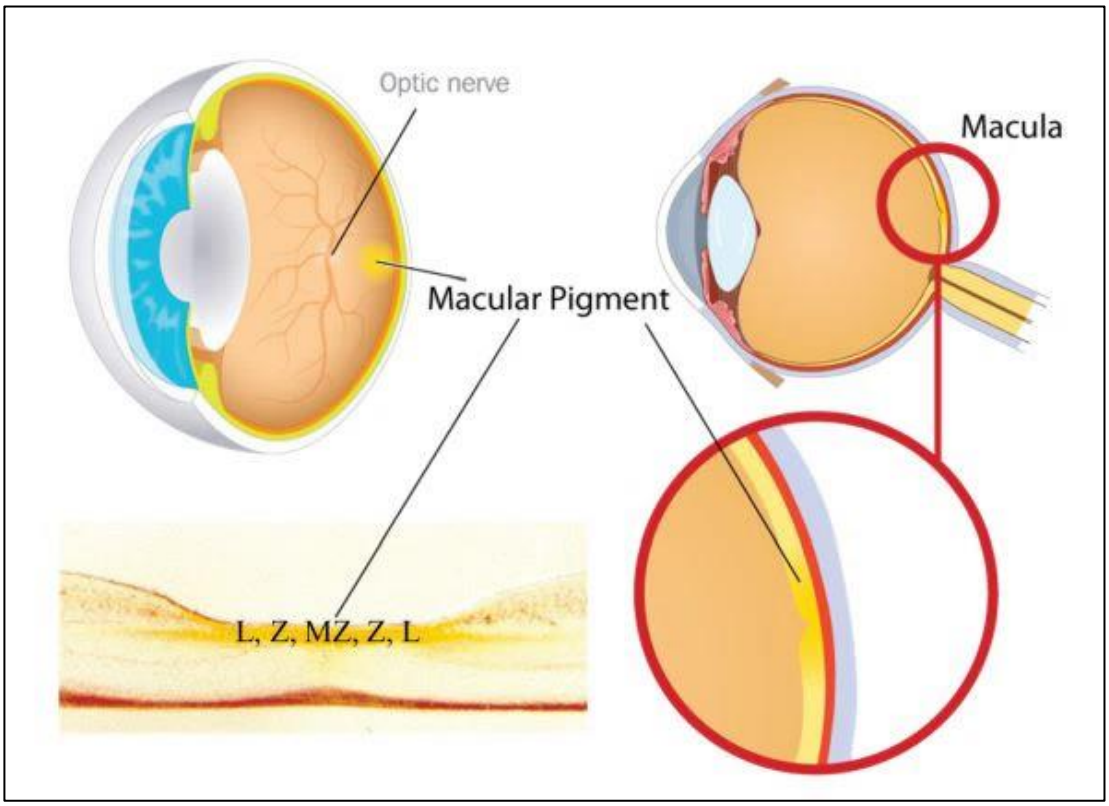

Figure 1.11: Location of macular pigment.[47, 49, 53] Image courtesy of Professor Max Snodderly, Austin, USA, and Professor John Nolan, Waterford, Ireland.

The fovea, the centre of the macula, is a small, central pit composed of closely packed cones and is responsible for almost all of our useful day (photopic) vision.[54] Only primates (i.e. humans and nonhuman primates) accumulate concentrations of L and Z at the fovea.[55] The highest concentration of MP has been observed in the Henle fibre layer (fovea) and inner nuclear layer (parafoveal) of the retina, with its distribution varying greatly between human individuals (200-900µm).[56]

L is dominant in both the diet (L:Z ratio of 7:1 to 5:1)[57, 58] and serum of humans (L:Z ratio of 4:1 to 2:1).[39, 59, 60] The ratio of L to Z varies across the unique structure of the fovea; Z is concentrated in the central fovea (ratio of L to Z is approximately 1:2.4), whereas L is concentrated in the periphery (ratio of L to Z is approximately 2:1) (Figure 1.12).[55] Furthermore, Bone and Landrum discovered that about 50% of Z in the retina is present as the stereoisomer of Z, MZ.[50, 61] Z and MZ accumulate at the centre (fovea) region of the macula where cone density is highest and risk of oxidative damage is greatest (Figure 1.13). Johnson *et al.* reported that MZ was the biochemical conversion of L (not Z) by means of oxidation and reduction.[62]

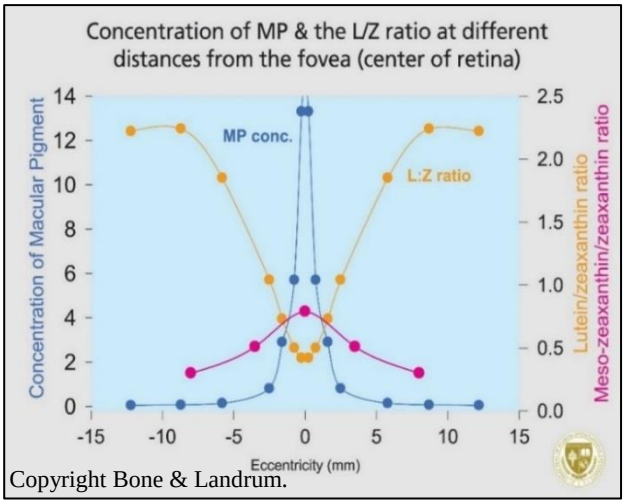

Figure 1.12: Graphical plot of the concentration of the macular pigment (MP) in the human retina as calculated from HPLC measurements (blue line). The lutein/zeaxanthin (L/Z) ratio varies by a factor of more than 4 over this narrow range of eccentricity (yellow line). The L/Z ratio reaches a minimum in the central macula where meso-zeaxanthin reaches highest levels and is approximately 50% of the total zeaxanthin present (pink line).[52]

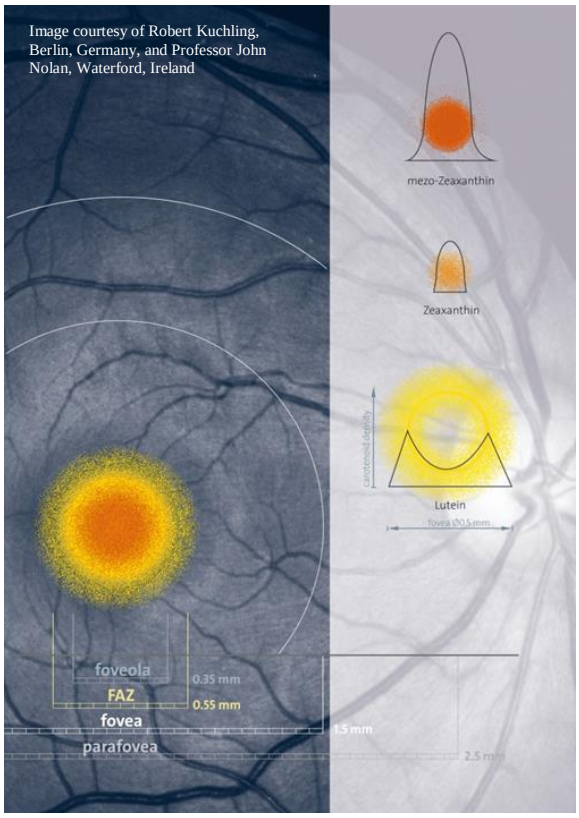

Figure 1.13: Location of lutein, meso-zeaxanthin and zeaxanthin at the macula.

The mechanism to explain the distinct distribution of xanthophylls remains unclear. However, the isolation of two xanthophyll-binding proteins, GSTP 1 and the StAR D3, (like L and Z are not uniformly disturbed) has suggested that these proteins may function as “transporters" of L and Z within the retinal layers.[63, 64] Genetic factors may also influence the ability to accumulate MP (see later discuss in Section 1.6.4).

1.6.2 Functions of MP

Although there are many hypotheses to explain the accumulation of MP, it is still unclear why these particular carotenoids co-exist in the retina over other micronutrients circulating in blood.[52, 65, 66] Of the 50 dietary carotenoids that are absorbed by the human body, only L, Z and MZ accumulate in the macula, reflecting an exquisite degree of biological selectivity.[49, 61] As MP's constituent carotenoids are found at uniquely high concentrations in the macula, it is believed that their purpose is two-fold: 1) photo-protection[67] and 2) antioxidant activity within the eye.[68] MP's constituent carotenoids have been shown to enhance visual function in diseased[69, 70] and non-diseased eyes[71] and reduces the risk of visual loss in, and progression of, AMD,[72] the leading cause of blindness in adults over the age of 65 years.[73-75]

The light absorbance spectrum of MP peaks at 460nm (Figure 1.14) and therefore this optical filter has the capacity to absorb/filter high energy blue light before it reaches the photoreceptors.[76] This light-filtering process minimises chromatic aberration (de-focused light) and light scatter, contributing to an improvement in visual function.[76] Indeed, this has been confirmed by our group as part of the Central Retinal Enrichment Supplementation Trials (CREST).[77]

MP's protective function lies in its antioxidant potential. As previously suggested, MP's constituent carotenoids are natural antioxidants and as a result can reduce oxidative stress in the retina (in vivo), where light and oxygen are abundantly present.[52] ROS are produced by absorption of ultraviolet (UV) and blue light.[78] The neutralisation of these ROS are relevant to pathogenesis of AMD (see later in discussion on AMD). The differences in ring type and hydroxyl group orientation in the macular

carotenoids determine their antioxidant properties, which are likely dependant on the size of their chromophore and their ability to form specific aggregates.[79]

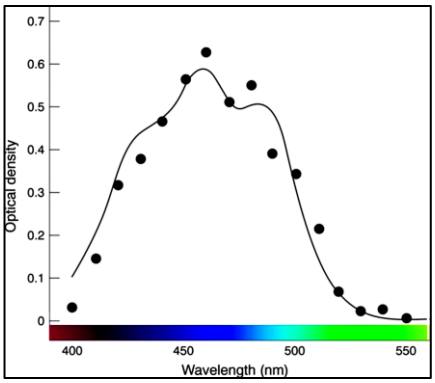

Figure 1.14: Absorbance spectrum of macular pigment. Image courtesy of Professor John Nolan.

L is orientated both parallel and perpendicular to the phospholipid bi-layer of the cell membrane, while Z and MZ are orientated perpendicular only. For the reasons mentioned above, L is considered a more efficacious filter of short-wavelength (blue) light when compared to Z and MZ.[80] Z and MZ exhibit greater antioxidant potential than L. Of note, however, these carotenoids work synergistically to optimise their antioxidant and light-filtering potential at the macula,[79] and it is for these synergistic advantages that we believe the retina accumulates these pigments in equal concentrations at this specialised tissue (ratio of 1:1:1).

While there is sufficient evidence of MP's role mentioned above, it has also been suggested these carotenoids influence the immunological and inflammatory responses in the eye as well as other areas of the body.[51] It has also been suggested that

drusen formation includes inflammatory and immune pathways,[81] and these pathways appear to be a key part of AMD pathogenesis.

The complement system, part of the immune system, promotes inflammation and clears foreign and damaged cells. The complement system consists of proteins (i.e. complement factors) that are normally inactive but can trigger immune functions such as phagocytosis, inflammation and membrane attack. Recently, Tian *et al.* reported that L supplementation significantly reduced circulating levels of the complement factors-D, C5a and C3d in early AMD patients when compared to a placebo group.[30, 82] It has also been speculated that high concentrations of plasma L in men and women from an area of Southern France may explain the lower incidence of coronary heart disease (CHD) when compared to an area in Northern Ireland.[83] CHD is known as an inflammatory disease and the macrophages within the plaques associated with tissue damage activate the immune system (e.g. complement factors). Of note, this study compared the blood cardiovascular risk factors and carotenoid concentrations in two areas: Toulouse, France and Belfast, Northern Ireland. The authors reported that the protective effects of L (through the intake of fruits and vegetables, which were correlated to plasma concentrations) prevented the activation of damaging complement factors in the blood. However, further work is required to understand the role of carotenoids and exact mechanism of action in reducing the effect of inflammatory makers in AMD and CHD.

1.6.3 Sources of L, Z and MZ

Approximately 78% of dietary L and Z is sourced from vegetables.[84] L, the most dominant xanthophyll carotenoid in the diet, is commonly found in dark-green leafy vegetables (spinach, kale, collard greens and broccoli), while orange peppers, corn and corn products are major dietary sources of Z.[85] Relatively high levels of L and Z are also present in asparagus, green beans and zucchini.[84] Perry *et al*. quantified the carotenoid concentrations in commonly consumed fruit and vegetables as shown in Table 1.1. Other databases such as the United States Department of Agriculture (USDA) nutrient database and other studies provide additional xanthophyll content in foods.[84, 86] Eggs also contain L and Z, and the fats present in the eggs enhance the bioavailability of these macular carotenoids.[87] More generally, cooking or ingesting macular carotenoids with dietary fat or oil increases the bioavailability of L and Z.[88] It was originally suggested that retinal MZ was non-dietary in origin and generated solely as a metabolite of retinal L (in animal models).[62, 89, 90] However, this view has since been refuted as its presence has been detected in the skin of trout, sardine and salmon, and in the flesh of trout.[91] Also, Bone *et al*. proved MZ can be absorbed into the serum and transported to the macula after carotenoid supplementation.[92]

As consumers become more aware of their health and more conscious about optimizing their diets, future studies are required to investigate the nutritional quality (e.g. carotenoid concentrations) and the impact of cultural practices (conventional and organic), post-harvesting handling and processing techniques of fruits and vegetables. As previously mentioned, carotenoids play a vital role in plants and animals, the most significant aspect being their antioxidant and light filtering properties. However, several

factors such as soil conditions, fertilizer applications and the cultivar can directly and indirectly affect the nutritional composition of the crop. Therefore, best management practices need to be identified to ensure that food is safe and nutritious for the consumer.

Table 1.1: Lutein and zeaxanthin concentrations in fruit and vegetables (μg/100 g).[85]

Fruit/ Vegetable	**Lutein (*trans*)**	**Zeaxanthin (*trans*)**
Artichoke heart	62	15
Asparagus, cooked	991	0
Broccoli, cooked	772	0
Brussel sprouts, cooked	155	0
Cilantro	7703	0
Cucumber	361	0
Endive	399	3
Green beans, cooked from frozen	306	0
Kale, cooked	8884	0
Kiwi	171	0
Lettuce, iceberg	171	12
Lima bean, cooked	155	0
Orange juice	33	26
Parsley	4326	0
Pepper, green	173	0
Pepper, orange	208	1665
Pepper, yellow	139	18
Scallions, raw	782	0
Scallions, cooked in oil		2488
Spinach, cooked	12,640	0
Spinach, raw	6603	0
Squash, yellow, cooked	150	0
Zucchini, cooked with skin	1335	0

An average western diet contains 1.3-3 milligrams (mg) per day of combined L and Z.[93, 94] Of note, the accuracy of the daily intake of carotenoids is highly dependent on the population studied and method of dietary assessment. MZ is unlikely to be found in a typical (un-supplemented) diet; however, it has been detected, *albeit* in small quantities, in fish and eggs from hens fed MZ.[89, 95, 96] In some cases, we do not obtain

enough of these nutrients from our diet, and it is for this reason, that L, MZ and Z have been formulated into oil suspensions and consumed as a dietary supplement. Supplementation with the macular carotenoids for eye health has increased substantially in the last twenty-five years, since the recommendation from optometrists and ophthalmologists that individuals with a family history of AMD, individuals afflicted with early AMD, and individuals with low levels of MP should consume an antioxidant supplement containing L, Z and/or MZ.

As L co-exists with other carotenoids in fruits and vegetables, the isolation and purification process for large quantities is expensive and time consuming. The petals from the marigold flower (*Tagetes erecta* L.) are a rich source of L and have very few other carotenoids present (i.e. approximately 5% of all-*trans* and *cis* isomers of Z).[44, 97] This flower is grown as a crop in Mexico, Ecuador, Spain, India, Peru, and China for analytical standards, dietary supplements and as a colour additive in foods. A number of patents are published for the isomerisation process of L (and L esters) into Z or MZ.[98, 99]

1.6.4 Determinants of MP

Beyond the dietary intake of foods mentioned above, determinants of MP include genetics, tobacco use, obesity, cumulative exposure to light, ethnicity and serum concentrations of L and Z.[100-106] Genetic variation together with non-dietary determinants of MP are likely to contribute to the variability between individuals and their response to dietary intake of the macular carotenoids. Non-dietary determinants account for 12-25% of the variation in MP.[101, 103] However, genetic factors may account

for 67% of variation in MP[104] and 27% of variation in MP response to supplementation of L and Z.[105] Variants in genes related to xanthophyll binding in retina (GSTP1), lipid and/or carotenoid absorption (SCARB1), HDL transport (ABCA1, ABCG5, LIPC), carotenoid cleavage (BCO1), omega-3 fatty acid status (ELOVL2, FADS1, FADS2), and genes related to maculopathies (RPE65, ALDH3A2) have been associated with MP levels in women from the Carotenoids in Age-related Eye Disease Study (CARDES).[106]

1.6.5 Techniques for measuring MP and its constituent carotenoids in human tissue and serum

Currently, there are a variety of different techniques (*in vivo* [i.e. within the living] and *ex vivo* [i.e. out of the living]) available for measuring MP and MP's constituent carotenoids (L, MZ and Z) in humans and animals. *In vivo* techniques such as heterochromatic flicker photometry (HFP) (MP optical density), Raman resonance spectroscopy (MP optical density) and fundus autofluorescence (MP optical density). *Ex vivo* techniques include HPLC (plasma and/or tissue) and mass-spectrometry (MS) (plasma and/or tissue). Dietary intakes of L, Z and MZ are typically collected through food frequency questionnaires (FFQ) or dietary screeners. It is debateable which technique should be deemed the "gold standard" however, it is important to note that each technique has its own advantages and limitations. For example, advantages of HFP (MP optical density) include: 1) non-invasive, 2) no pupil dilation required, 3) proven validity, and 4) good test-retest reliability in many subject populations. However, limitations of HPF include: 1) some subjects find HFP difficult to carry out, 2) long

testing time, and 3) unsuitable for children, individuals with learning difficulties or individuals with insufficient visual acuity.[107] The advantages of HPLC analysis of biological samples (blood and/or tissue) include: 1) frequently used as biomarker of fruit and vegetable intake and 2) simultaneous measurement of various carotenoids; however, the disadvantages include 1) expensive and specialised laboratories required, 2) invasive (i.e. venepuncture or biopsy) and 3) carotenoid levels are also influenced by recent dietary intake, seasonal variation, and physiologic factors such as absorption and metabolism.[108] The advantages of food frequency questionnaires include 1) relatively low administrative costs and time and 2) the ability to assess usual and longer term intake; disadvantages include 1) inaccuracy of absolute nutrient values, 2) lack of detail regarding specific foods, and 3) general imprecision.[109]

1.6.6 Safety of L, Z and MZ

No adverse effects have been noted in the scientific literature for L, MZ and/or Z.[110-113] In 2004, the Joint Food and Agriculture/WHO Expert Committee on Food Additives set the acceptable daily intake (ADI) for L (from *Tagetes erecta*) and synthetic Z at 0-2 mg/kg.[114] For example, a person weighing 70 kg could safely consume 140 mg of combined L and Z. However, at the most recent meeting (2014), the committee established a temporary ADI as not specified, which remains tentative until the specifications for L esters are finalised.[115] It has been reported MZ had no acute toxicity and no genotoxicity effects in a 90-day feeding study in rats. The ADI for MZ was set at 3 mg/kg.[116] In summary, the ADI of macular carotenoids is a far greater dose than what would be consumed in a ‘normal’ diet or used in dietary supplements.

Nevertheless, a lack of clarity remains with respect to the safety and recommended intake levels of antioxidant (e.g. macular carotenoids) supplementation over long periods of time in patients with AMD or at risk of developing AMD.[117] While Khachik *et al*. reported that supplementation with high doses of L and/or Z in monkeys did not cause ocular toxicity,[118] Choi *et al.* reported crystalline maculopathy in a patient (free of macular degeneration) who had an exceeding high daily ingestion of lutein in the previous eight years.[119] Furthermore, carotenoid breakdown products have been shown to be toxic to RPE cells.[120] A scenario to consider is the findings from randomized controlled trials (Beta-Carotene and Retinol Efficacy Trial and Alpha-Tocopherol, Beta Carotene Cancer Prevention Study) in which beta-carotene supplementation in smokers and individuals with occupational exposure to asbestos was associated with an increase in lung cancer incidence.[121-123] Evidence for such risks of L, Z and/or MZ supplementation over long periods of time will become available as more individual follow the advice given by their eye care professionals regarding antioxidant supplementation. Therefore, attention should be placed on establishing recommend intakes or dietary guidance for the inclusion of bioactive compounds (e.g. macular carotenoids) in foodstuff and/or supplements.[124-126]

1.6.7 MP (and MP's consistent carotenoids) and diseases

Diseases related to oxidative stress may be associated with lower levels of MP (and MP's constituent carotenoid concentrations). MP (and MP's consistent carotenoids) have been studied in diseases such as: AMD,[127, 128] glaucoma,[129] cataracts,[130, 131] AD,[132] and CVD.[133-135] The finding of lower MP in patients with these diseases may be

explained by a multiple of factors such as poor diet and/or absorption and/or bioavailability of L, Z and MZ in blood and host tissues. Additional research is warranted to further study MP (and MP's consistent carotenoids) and evaluate the strength to associated diseases.

1.7 Age-related macular degeneration (AMD)

In 1875, Hutchinson and Tay described 10 cases of whitish spots in the macula of older adults, which they termed "senile macular degeneration".[136] Three of these cases were sisters who reported visual loss and a family history of poor sight, although the cause was unknown. The accumulation of yellow or white extracellular material (drusen) builds up between the Bruch's membrane and the retinal pigment epithelium (RPE) as a normal part of ageing. However, the presence of numerous tiny or larger drusen at the macula is a hallmark of what has been defined as AMD.[137] AMD is a disease of the macula that, in its advanced stage, results in the loss of central vision.[138] Early (non-advanced) AMD is characterised by drusen and/or pigmentary abnormalities, whereas the late (advanced) form of AMD is visually consequential and can be classified as atrophic (geographic atrophy [GA] or dry) or neovascular (choroidal neovascularization [CNV] or wet).[139]

1.7.1 Prevalence and incidence of AMD

AMD is the leading cause of irreversible blindness in the older population, especially in developed countries.[140-142] It has been well established by many international

epidemiological studies that our eyes exhibit an age-related decline resulting in vision loss and eye disease.[143-145] The prevalence of AMD increases with increasing age and the number of new cases is increasing over time. Pooling data from 39 studies (and applying a Hierarchical Bayesian approach), Wong *et al.* estimated the global prevalence of any form (i.e. early or late) of AMD to be 8.7% in those aged 45 to 85 years.[146] The global projection of people with AMD is estimated at 196 million by 2020, further increasing to 288 million by 2040.[146] Although an ageing population is one of the success stories of modern society, it does generate new challenges (public policy, financial issues, and the wellbeing of the elderly) for many developed countries such as Ireland. In the Republic of Ireland (ROI), Kelliher *et al*. estimated that AMD accounted for 25% of all blind registration in Ireland (2006).[147] Recently, Akuffo *et al*. estimated that the overall prevalence of any form (i.e. early or late) of AMD among adults aged 50 years or older in the ROI is 7.2% (census-weighted).[73]

1.7.2 Classification of AMD

Different classification and grading system for AMD, such as the Wisconsin Age-related Maculopathy Grading System, International Classification and Grading System for Age-Related Maculopathy and Age-related Macular Degeneration, The Age-Related Eye Disease Study Severity Scale, Age-Related Eye Disease Study Simplified Severity Scale, and Clinical Classification System of Age-related Macular Degeneration, have been established and utilised in epidemiological and clinical studies.

A modified version of the International Classification and Grading System for Age-Related Maculopathy and Age-related Macular Degeneration was used in Chapter three and four. In summary, early AMD was defined as the presence of drusen and RPE pigmentary abnormalities (hyperpigmentation and/or hypopigmentation). Late AMD was defined as GA (dry AMD) or neovascular AMD (wet AMD).[139] GA was defined as the presence of a roughly round or oval area (at least 175μm) of hypopigmentation or depigmentation with clearly visible choroidal vessels. Neovascular AMD was defined as the presence of any of the following characteristics: serous and haemorrhagic RPE detachment, retinal haemorrhage, scar/glial/fibrous tissue, and hard exudates (not associated with other retinal vascular disease).[139]

1.7.3 Aetiopathogenesis of AMD

While the exact cause of AMD remains unclear, it has been suggested AMD is a result of many contributing factors, with influences from genetic, lifestyle and environmental factors contributing in the development of the disease. It has also been hypothesized that changes in the macular region (Bruch's membrane, RPE, and choriocapillaries) contribute to the initiation and/or progression of AMD. At least three of the processes (genetic variation, inflammation and tissue damage) contributing to AMD are discussed below.

It seems very likely that genetic predisposition is involved in the development of AMD. Multiple studies have assessed the role of genetic variants on risk and progression of AMD.[148-150] Common variants in genes (ABCA4, APOE, ARMS 2, C3,

HTRA1 and VEGFA), which encode proteins with roles in inflammation, dyslipidaemia, oxidative stress, and tissue dysfunction, have been implicated with AMD.[151] While, a variant (Y402H) within the complement factor H gene has shown a strong association with the pathogenesis of AMD. [152-154] Furthermore, the joint effect of a high genetic risk of Y402H and a poor lifestyle (diet, physical activity and smoking) may attribute to the burden of AMD.[155] Genetic variations within SCARB1 have implicated a role for cholesterol metabolism and L in AMD pathogenesis (a common pathway shared with CVD).[156, 157]

AMD is characterised by loss of photoreceptors and RPE cells.[158] The free radical theory of ageing may in part explain the aetiopathogenesis of AMD. The driving force of age-related diseases is the cumulative tissue damage caused by ROS. The generation of ROS increase in response to irradiation, ageing, inflammation, or environmental stress such as pollution, cigarette smoke, and pesticides. The retina is an ideal environment for the generation of ROS, because of its high oxygen demand and consumption of oxygen, its high proportion of polyunsaturated fatty acids (PUFAs), and its exposure to visible light (retinal irradiation). PUFAs are susceptible to free radical damage as their conjugated double bonds are a convenient source of hydrogen. In addition, several inflammatory cytokines (vascular endothelial growth factor [VEGF], interleukin [IL]-6 and angiotensin II) have been reported to cause severe retinal damage.[159-161]

Furthermore, retinal chromophores such as lipofuscin (yellow-brown pigment granules) are known as ageing pigments. These pigments are composed of lipid-containing residues of lysosomal digestion, which may disrupt RPE cellular activities

by reducing functional cytoplasmic space,[162] inducing oxidative damage of surrounding tissue[163] or limit digestion of intra/extracellular material.[164] In the retina, lipofuscin is partly derived from oxidative damage to the outer segments of photoreceptors. The primary fluorophores of lipofuscin are bisretinoids formed as a by-product of the visual pigment cycle, such as all-*trans*-retinal dimer linked to phosphotidyl ethanolamine.[165] In addition, light of shorter wavelengths (visible light) has been shown to damage RPE and photoreceptors.[166]

1.7.4 Risk factors for AMD

Several epidemiological studies have investigated possible risk factors for AMD, and found positive association with increasing age,[167-174] obesity (BMI),[168, 174-176] tobacco use,[168, 171, 173, 177, 178] family history of AMD,[179, 180] hypertension,[141, 167, 168, 174, 175, 181] and increased cholesterol levels.[182, 183] Putative risk factors for AMD, which remain inconsistent with the literature include light iris colour,[184-186] refractive error,[167, 173, 187] oestrogen[173, 181, 185] and alcohol consumption.[188-190] However, increasing age, tobacco use and family history of AMD have been deemed as established risk factors for AMD.[191]

1.7.5 Impact of AMD

AMD does not result in complete blindness, but the loss of central vision can interfere with an individual's quality of life and negatively impact their social engagement.[192]

Even a small decline of visual impairment has substantial adverse impacts on the quality and length of life. For instance, vision of 6/12 (80%) or less is associated with the following: loss of driving licence, increased risk of falls, hip fractures and depression, admission to residential homes years before a similar age group with normal vision, loss of social independence, and a reduced ability to enjoy healthy and independent ageing.[193]

Many AMD sufferers have difficulties carrying out activities of daily living such as shopping, managing finances, preparing meals, using the telephone and light housework.[194] AMD takes its toll on a person's psychological wellbeing with many AMD patients reporting emotional distress levels comparable to that of people battling other serious chronic illness.[194] A reduction in the number of hobbies, lack of independence and social isolation, can cause an increase in the rate of depression in AMD suffers.[195] As with many chronic diseases, AMD can also lead to financial hardship, especially since this age group (65 years and older) already struggles with very limited resources. Many AMD patients feel like a burden and are often pressured into residential homes by their family members. In addition to visual impairment as a result of AMD, many people report comorbidities such as CVD, hip replacements, cancer, stroke and arthritis.[196] All these negative issues, which are associated with AMD, can greatly reduce an individual's quality of life.[195]

1.7.6 Costs associated with AMD

The cost implications associated with visual impairment and vision loss can be a serious healthcare burden and cannot be overlooked. The Global Burden of Disease (1991-2001) reported that vision loss as the sixth most important cause of disability.[197] The cost of vision loss and impairment may be classed as direct and indirect. The indirect costs include the loss of earnings (by the patient), the cost of caregivers and nursing homes and other costs (e.g. transport). Direct costs include hospital care, outpatient and office visits, optometry costs, drugs and other direct medical expenses. The overall cost of blindness and vision impairment in ROI was approximately €386 million in 2010, and expected to rise to €449 million by 2020.[198] The cost of AMD to the Irish economy is estimated to be in the region of €133 million per annum.[198]

1.7.7 Treatment for or prevention of AMD

While there is no cure for AMD at this time, there are widely accepted treatment options. Neovascular late (wet) AMD is treated by anti-VEGF therapy, which has been shown to dramatically reduce the risk of visual loss.[199] Patients with early AMD or late atrophic AMD are encouraged to reduce risk of disease progression by changing their lifestyle factors such as quitting smoking, adopting a healthy diet and/or taking dietary supplements containing antioxidants. The Age-Related Eye Disease Study (AREDS), a multi-centre supplementation trial carried out in the United States, which commenced in 1992, evaluated the role of antioxidant supplementation in patients with intermediate AMD.[200] The antioxidant supplement in this study contained 500 mg of vitamin C, 400

IU of vitamin E, 15 mg of beta-carotene, and 80 mg of zinc. AREDS found a 26% risk reduction for progression to advanced AMD.[200] Of note, L and Z were not commercially available in supplement form at the time of the study. The subsequent AREDS 2 study, which commenced in 2006, examined several changes to the formulation including the addition of omega-3 fatty acids and the replacement of beta-carotene with the xanthophylls, L and Z (which were commercially available at this time). AREDS 2 reported a reduction in participants who progressed from intermediate AMD to advanced AMD, if they received antioxidant supplements (containing L and Z).[72] Studies have also evaluated the role of dietary supplements, containing L, Z, and/or MZ in conjunction with other antioxidants, in preserving and/or improving visual acuity,[201-203] contrast sensitivity,[66, 184, 185] photostress recovery[204] and glare disability[201] in subjects with AMD.

1.8 Macular carotenoids and visual function

A number of intervention studies have suggested that supplementation with the macular carotenoids (L and/or Z and/or MZ) could improve visual acuity[71] and contrast sensitivity[205-207] in young, healthy subjects and contrast sensitivity[77] in subjects free of retinal disease.

1.9 Epidemiological studies of L and Z, and AMD

In the last 25 years, there has been a considerable amount of research dedicated to studying plasma concentrations of L and Z in humans. A number of studies have investigated the relationship between plasma concentrations of L and Z and dietary intake, while others associate plasma concentrations of L and Z with risk factors for certain diseases such as metabolic syndrome,[208] breast cancer,[209] cystic fibrosis,[210] AMD[211] and cardiovascular disease.[212]

Table 1.2 summarizes cross-sectional studies designed to investigate a possible relationship between AMD and MP and/or serum concentrations of MP's constituent carotenoids. This review includes population-based and case-control studies from different ethnicities, disease status and age groups. These studies will be used as comparison to the TILDA plasma carotenoid database, which was uniquely available for this thesis, as it is crucial to develop a solid understanding of plasma concentrations of L and Z in various populations.

In 2009, Connell *et al*.[213] reviewed a number of large epidemiology studies investigating the relationship of serum concentrations of carotenoids with risk for AMD, including the National Health and Nutrition Examination Survey (NHANES) III[214] and Pathologies Oculaires Liess a L'Age (POLA0).[215] CAREDS investigated the prevalence of AMD and lifestyle factors in older women.[103, 211] Mares *et al*. (CAREDS) reported that MP optical density was directly related to dietary intake and serum concentrations of L and Z, while Moeller *et al*. concluded that diets rich in L and Z may protect against AMD in women younger than 75 years old. Of interest, the Beaver Dam

Eye study (BDES) reported that serum concentrations of L and Z were not correlated with AMD.[216] Although the Rotterdam study had access to serum carotenoid data, no publications have emerged assessing their association with AMD status.[171] However, authors did report that a high dietary intake of nutrients (including L and Z) reduced the risk of early AMD in those with a family history of the disease.[217] The Eye Disease Case Control Study (EDCCS) examined the risk factors for neovascular late AMD, including serum carotenoid concentrations, and reported that decreased risk of neovascular AMD was associated with higher serum concentrations of carotenoids.[173] The Muenster Ageing and Retina study (MARS) evaluated the association of serum concentrations of L and Z and AMD in a case-control analysis of baseline data.[218] Dasch *et al*. reported that serum concentrations of L and Z were not related to the prevalence of AMD; however, this may have been confounded by L and Z supplementation in this population. Gale *et al*. found that the risk of AMD was significantly higher in participants with lower plasma Z, but not L.[219]

Table 1.2: Summary of cross-sectional studies designed to investigate a possible relationship between age-related macular degeneration and macular pigment and/or serum concentrations of macular pigment's constituent carotenoids.

First Author, publication year	Period of data collection	Sample size	Type of study	Correlates under investigation	Use of L and/or, Z and/or MZ containing supplements	Principal AMD finding
EDCC Study Group, 1993[220]	1986-1990	968	C-C	Serum L and Z (combined)	Data not recorded	*Inverse relationship with serum L and Z*
Mares-Perlman *et al*., 1995[216]	1988-1990	334	C-C	Serum L and Z (combined)	Data not recorded	No relationship identified
Mares-Perlman *et al*., 2001[214]	1988-1994	8,222	P-B	Serum L and Z (combined)	Data not recorded	*Inverse relationship with serum L and Z***
Sanders *et al*., 1993[221]		130	C-C	Serum L	Data not recorded	No relationship identified
Moeller *et al*., 2006[37]	1994-98 & 2001-04*|	1,787	P-B	Serum L and Z	Data not factored	No relationship identified
Gale *et al*., 2003[219]	1996-1997	380	P-B	Serum L and Z	Data not recorded	*Inverse relationship with serum Z*
Delcourt *et al*., 2006[215]	1996-1997	640	P-B	Serum L and Z	Data not recorded	*Inverse relationship with serum L and Z*
Bernstein *et al*., 2002[222]	2000-2001	201	C-C	MP (Raman)	Data recorded and factored into analyses	*Inverse relationship with MP*
Dasch *et al*., 2005[218]	2001-2003	910	C-C	Serum L and Z	Data recorded and factored into analyses	No relationship identified
LaRowe *et al*., 2008[223]	2001-2004	1698	P-B	MP (HFP)	Data recorded and factored into analyses	No relationship identified
Beatty *et al*., 2001[128]		18	C-C	MP (HFP)	Data not recorded	*Inverse relationship with MP*
Bone *et al*., 2001[127]		112	C-C	MP (HPLC)	Data not recorded	*Inverse relationship with MP*
Simonelli *et al*., 2002[224]		94	C-C	Serum L and Z (combined)	Data not recorded	No relationship identified
Wüstemeyer *et al*., 2002[225]		20	C-C	MP (SLO)	Data not recorded	*Inverse relationship with MP*
Dietzel *et al*., 2011[226]	2003-2006	369	P-B	MP (AF)	Data recorded and factored into analyses	No relationship identified
Ciulla *et al*., 2004[227]		110	C-C	MP (HFP)	Data not factored into analyses	No relationship identified
Obana *et al*., 2008[228]	2005	197	C-C	MP (Raman)	Data not factored into analyses	*Inverse relationship with MP*
Cardinault *et al*., 2005[229]		55	C-C	Serum L and Z	Data not recorded	No relationship identified
Michikawa *et al*., 2009[230]	2005-2006	722	P-B	Serum L and Z (combined)	Data not recorded	No relationship identified.
Zhou *et al*., 2011[231]	2007-2008	263	C-C	Serum L and Z	Data recorded and factored into analyses	*Inverse relationship with serum Z*
Tsika *et al*., 2011[232]		102	C-C	MP (HFP)	Data recorded and factored into analyses	*Inverse relationship with MP*
Ren *et al*., 2015[233]	2012	225	C-C	MP (HFP)	Data not recorded	No relationship identified
Kaya *et al*., 2012[234]		181	C-C	MP (Fundus)	Data recorded and factored into analyses	*Inverse relationship with MP*
Puell *et al*., 2013[235]		49	C-C	MP (Metropsis)	Data not recorded	No relationship identified

Publications organized by publication date in the absence of authors reporting period of data collection; principal finding is italicized when an inverse relationship is reported. EDCC Study Group, Eye Disease Case-Control Study Group; Type of study: C-C, case-control study; P-B, population-based study; Correlates under investigation: serum lutein (L) and zeaxanthin (Z), concentrations measured by high performance liquid chromatography (HPLC); MP, macular pigment; HPF, heterochromatic flicker photometry; fundus, spectral fundus reflectance; AF, autofluorescence; Raman, resonance Raman spectroscopy; Metropsis, cathode ray tube-based Metropsis psychophysical vision test; Densitometer, macular densitometer; SLO, confocal scanning laser ophthalmoscope; Use of L and/or, Z and/or MZ supplements: Data not recorded; supplement data not collected during study visit; Data not factored into analyses, supplement data was collected but not used in analysis between age-related macular degeneration (AMD) subjects and control subjects; Data recorded and factored into analyses, data of supplement use was collected during study and factored into analyse, exclusion of subjects consuming supplements, or confirmation that none of the subjects consumed supplements; *Serum concentrations of L and Z were collected in 1994-1998, however data on supplement use was collected 2001-2004; **inverse relationship, though marginal.

1.10 Determinants of L, Z and MZ

Determinants and correlates of plasma concentrations of L, Z and MZ are best classified as modifiable and non-modifiable. Although, dietary intake is the most obvious modifiable determinant of plasma concentrations of L, Z and MZ, non-modifiable determinants such as age, sex, genetics and ethnicity may also determine the circulating plasma concentrations of these nutrients.[236-238] Variants in genes related to xanthophyll binding in retina (STARD3), lipid and/or carotenoid absorption (SCARB1, CD36), HDL transport (ABCA1, ABCG5, ABCG8, NPC1L1, CETP), carotenoid cleavage (BCMO1), and genes related to maculopathies (RPE65) have been associated with serum concentrations L and Z in women from CARDES.[239] Other modifiable risk factors such as demographic, lifestyle and environmental factors (e.g. region, cholesterol, smoking status, and BMI) may also be strong predicators of plasma concentrations of L, Z and MZ.[238, 240, 241]

1.11 Purpose and rationale of current study

The absorption characteristics and antioxidant properties of the macular carotenoids (L, Z and MZ), which have been discussed in this chapter, give plausible reason to believe MP protects against AMD. The aim of this research was to study these macular carotenoids and try to understand what determines their concentrations in plasma in an attempt to discover lifestyle or environmental factors, which can be modified to increase their concentrations in plasma and ultimately promote retinal health.

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Chapter 2: Methods

2.1 The Irish Longitudinal Study on Ageing (TILDA)

2.1.1 Introduction

While the UK,[1] USA[2] and other developed countries[3] have established well-known large population-based studies on human ageing, Ireland had yet to embark on their own comprehensive and international comparable survey on older adult health. In 2009, researchers based in Trinity College Dublin in the ROI began a comprehensive longitudinal study on the health, economic and social status of Irish adults aged 50 years and over.[4] This landmark study is known as TILDA. In brief, TILDA was designed to investigate the factors that influence healthy ageing and is by far the most ambitious and largest ageing study of its kind in the ROI. This study will provide the foundation to develop new policies in healthcare, the financial sector and communities in Ireland and has the opportunity to implement innovative technology that will make ageing a better experience.

The two main designs in which the effect of ageing on a population can be measured are cross-sectional (measurements at same time point) and longitudinal (serial measurements). While TILDA is a longitudinal study, due to feasibility and timeframe of my research, baseline (wave 1) was the focus of this thesis.

In relation to this thesis, this study has the unique opportunity to assess the circulating plasma concentrations of L and Z and factors that determine the concentrations of these nutrients from a large randomly selected sample of the Irish population. Details of the methodology of TILDA are discussed below.

2.1.2 Target population

The target population for the TILDA study was the population of persons aged 50 years old or over who live in residential addresses in the ROI (i.e. excluding individuals living in long term care institution).[5]

2.1.3 Target sample

Based on statistical and resource factors, the TILDA target sample size to provide a national representation of individuals aged 50 years old or over in the ROI for baseline data was 8,000 participants.[4]

2.1.4 Sampling method

A nationally representative sample of community dwelling adults was drawn from the Irish Geodirectory, a current and comprehensive record of all residential addresses in the ROI created by 'An Post' (the Irish Postal Service) and Ordnance Survey. Residential addresses were selected by means of RANSAM (a random sampling design for Ireland), developed by the Economic and Social Research Institute.[6] Addresses were selected using a three stage process that has been previously reported by Kearney *et al.*:[5]

(i) All residential addresses in the ROI were assigned to one of 3,155 geographic clusters (500- 1180 addresses). From these clusters, a sample of 640 clusters were stratified by socio-economic status (per cent in professional/managerial occupations), age structure (per cent of population aged ≥50 years) and geographic location. Selection of clusters were based on the probability proportionate to size (i.e. the size being the estimated number

of addresses containing a person aged ≥50 years in the cluster) (see Figure 2.1);

(ii) 50 addresses were selected from the 640 clusters. The selected addresses were randomly assigned into two groups: a) an initial sample list of 25,600 addresses (40 addresses x 640 clusters,) for immediate recruitment and b) a reserve list of 6,400 addresses (10 addresses x 640 clusters);

(iii) all household residents aged 50 years and older were eligible to participate in the study (named as primary respondent). The spouses/partners (of any age) of primary respondents were also invited to participate (named as secondary respondent). The secondary respondents aged <50 years were interviewed mainly to provide additional household-level data and were not included in person-level analyses.

All residential addresses in the ROI had an equal probability of selection and all persons aged ≥50 years in each household were eligible (i.e. each person aged ≥50 years had an equal probability of selection).

In summary, the sampling method used in TILDA was designed to ensure that the selection of participants would be representative of the Irish population aged 50 years and over.

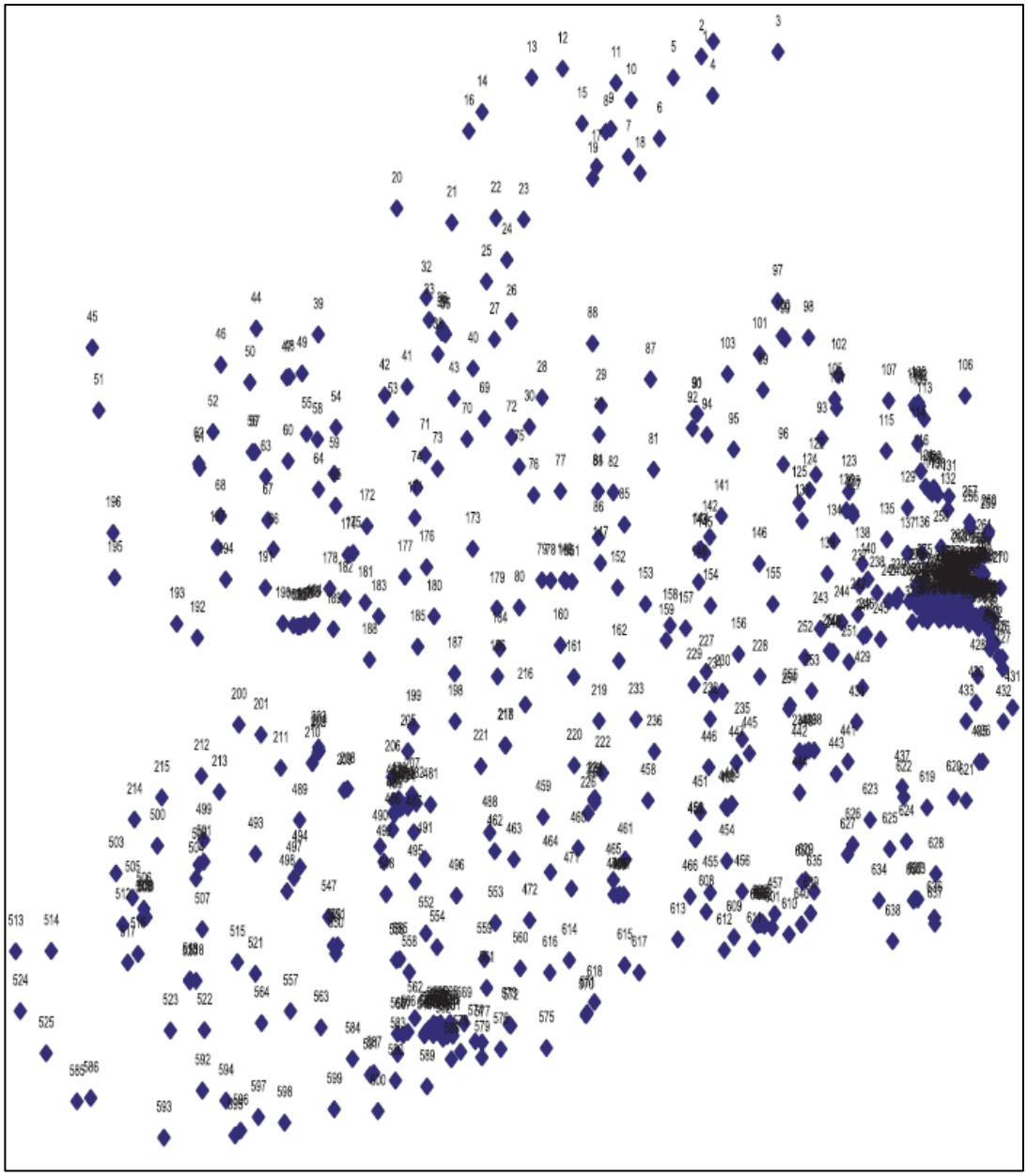


Figure 2.1: Map of the initial sample of Irish addresses chosen by means of the RANSAM sampling - location of the 640 clusters in the Republic of Ireland.[5]

2.1.5 Participant recruitment

An invitation to participate in the study was sent to selected households (25,600 addresses). The reserve list (6,400 addresses) was not used as the target sample size was

achieved using the first initial sample. This letter was followed-up by a home visit from a member of the TILDA field staff, which attempted to determine the eligibility of household members (aged ≥50 years). All individuals aged 50 years and over in each selected household were invited to be included in the study.

2.1.6 Study design

Eligible participants that took part in the TILDA study underwent a structured interview, completed a questionnaire every 2 years and were invited to attend a health assessment every 4 years as shown in Figure 2.2. Wave 1 was the foucs of this thesis.

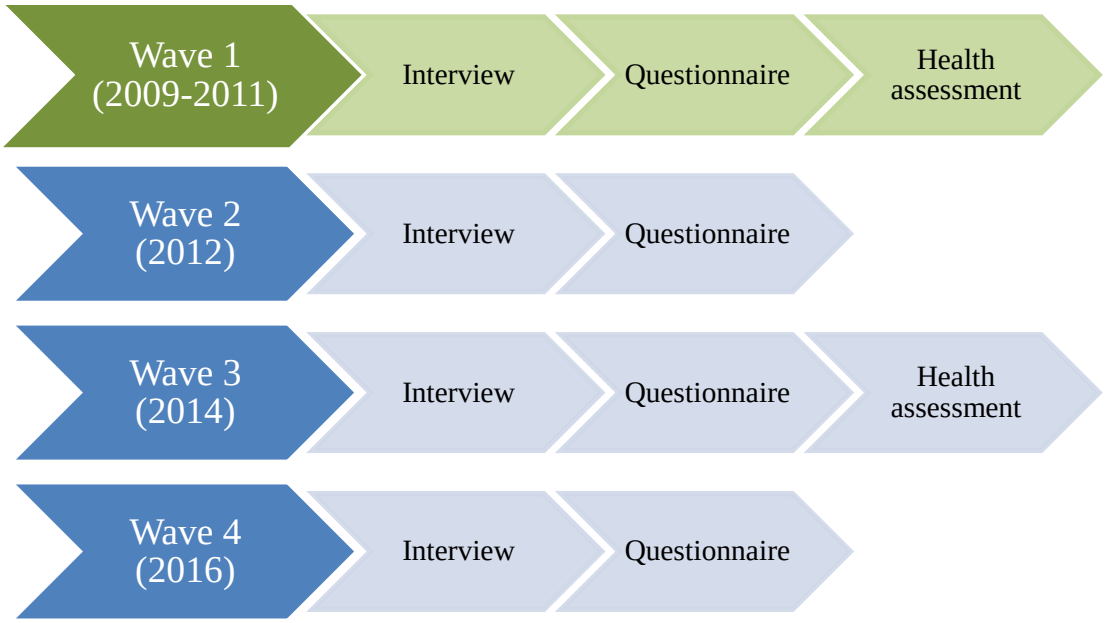


Figure 2.2: Timetable and flowchart of TILDA waves.

2.1.7 Ethics

This study was approved by the Faculty of Health Sciences Research Ethics Committee of Trinity College Dublin (Appendix A). Ethical approval for each wave of data collection is granted by the Trinity College Research Ethics Committee. All experimental procedures adhered to the tenets of the Declaration of Helsinki. Participants were required to provide written informed consent prior to participation in the study. Separate written and verbal consent was required to obtain and store blood samples from participants (for unspecified research purposes). As per the rules and regulations of Waterford Institute of Technology, this study was also approved by the local Ethics Committee at the Waterford Institute of Technology (Appendix B).

2.1.8 Funding

TILDA was funded by the Department for Health and Children, Irish Life and The Atlantic Philanthropies. Plasma carotenoid analysis was supported by Bayer, Ireland.

2.1.9 Components of the TILDA study

Interview

In each wave, participants completed a computer-aided personal interview (CAPI) carried out by a trained social interviewer in the participants' home. The interview was arranged for a time that suited the participant, which took about 90 minutes to complete. The interviewers collected information on demographics (education), social circumstance (children and helpers), sources of income and assets, physical and mental

health, employment and lifelong learning, planning for retirement and expectations, and behaviour help (smoking, alcohol and physical activity) (Table 2.1).

Table 2.1: Summary of TILDA wave 1 data collected in the Computer Assisted Personal Interview (CAPI) and Self-Completion Questionnaire (SCQ).

Demographic data	**Physical health**
Education (CAPI) *	Self-rated health (CAPI)
Childhood health (CAPI)	Limiting long-standing illness/disability (CAPI)
Migration history (CAPI)	Sensory decline (CAPI)
Marital status and marriage history (CAPI)	Cardiovascular disease (CAPI) *
Social circumstances	Non-cardiovascular disease (CAPI) *
Transfers to (and from) Children (CAPI)	Falls/fear of falling/steadiness (CAPI)
Transfers to (and from) Parents (CAPI)	Chronic pain (CAPI)
Act ivies of daily living (CAPI)	Incontinence (CAPI)
Helpers (CAPI)	Medical screening (CAPI)
Social connectedness (CAPI)	**Mental health**
Participation in social/recreation activities (CAPI)	Self-reported mental health (CAPI)
Relationship quality (CAPI)	Depression (CAPI)
Employment and Lifelong Learning	Life satisfaction (CAPI)
Employment situation (CAPI) *	Anxiety (SCQ)
Job history (CAPI)	Worry (SCQ)
Lifelong learning (CAPI)	Loneliness (SCQ)
Retirement and Expectations	Stressful life events (SCQ)
Planning for retirement (CAPI)	Quality of life (SCQ)
Expectations (CAPI)	**Cognitive health**
Income and Assets	Self-rate memory (CAPI)
Sources of income (CAPI)	Orientation (CAPI)
Assets (CAPI)	Word-list learning (CAPI)
Transport	Verbal fluency (CAPI)
Transportation (CAPI)	Prospective memory (CAPI)
Driving (CAPI)	**Behavioural health**
Medications (CAPI) *	Smoking (CAPI) *
Healthcare Utilization	Physical activity (CAPI) *
Ageing Perceptions (SCQ)	Alcohol (SCQ) *

* Variables of interest in this thesis

The interview collected detailed information on many aspects of the participants' lives. Details of the variables of interest in this thesis are discussed below. Age (years), sex (male/female), highest level of education (primary/none, secondary, and third level), geographic location (Dublin city/county, another city or town, and rural), alcohol consumption (number of drinks per week during the last 6 months), smoking status (never, past or current smoker), physical exercise derived from a modified version of the International Physical Activity Questionnaire-short form (inactive [low], minimally active [medium] and health enhancing physical exercise [high]),[7] self-report of a doctor's diagnosis of eye disease such as AMD, diabetic maculopathy, diabetic retinopathy, cataracts and glaucoma, family history of AMD (yes, no and don't know), self-reported high blood pressure (yes/no) and a list of medications taken on a daily basis (coded using Anatomical Therapeutic Chemical),[8] including food supplements (defined according to the Directive 2002/46/EC of the European Parliament and the Council of the European Union, 10 June 2002) were recorded for each participant. It is important to note that self-reported data is susceptible to bias, and this limitation must be taken into account when interpreting results here.

Self-completion questionnaire

The interview was followed by a short questionnaire, which included potentially sensitive questions that the TILDA interviewer left for the participant to complete and return via post or for collection by the interviewer. The self-completion questionnaire included questions about: quality of life, personality and values, social activities and

hobbies, quality of relationships, attitudes towards ageing, and accommodation and the local area (Table 2.1).

Health Assessment

After the interview, participants were invited to attend a comprehensive health assessment carried out by a team of trained nurses in one of two dedicated health centres (located in Dublin and Cork). If the participant was unable/unwilling to travel, a modified assessment was carried out in the participant's own home. The variables of interest in this thesis, as indicated in Table 2.2, were BMI, retinal photographs, MP, blood pressure, and venous blood samples. Full details of the methodology for these measurements are reported in Section 3.2 and 4.2. Some measurements such as retinal photography, MP, contrast sensitivity and visual acuity were only carried out in the health centre (i.e. measurements not carried out during the modified home assessment).

Participants were asked not to fast before the health assessment. However, if the participant did fast, consent and blood samples were obtained at the beginning of the health assessment. This allowed the participant to eat before completing the rest of the assessment; otherwise their performance in other measurements could have been adversely affected. If the participant (majority of sample) did not fast, blood was obtained at the end of the health assessment. The time of the participant's last meal was recorded in all cases.

A total of 25 ml of blood was collected into three vacutainers (one 5 ml Lithium Heparin tube and two 10 ml Ethylene Diamine Tetra-acetic Acid [EDTA] tubes). During the blood collection, one of the EDTA tubes was immediately covered in tinfoil

to protect the sample from light. This blood sample was dedicated to carotenoid assessment. All blood tubes were stored between 2-8°C up to 48 hours before they were delivered to the central laboratory in Dublin for immediate processing (lipid profile) or storage at -80°C. A complete lipid profile including total cholesterol (TC), high density lipoprotein (HDL), low density lipoprotein (LDL) and triglyceride was measured by a commercial laboratory.

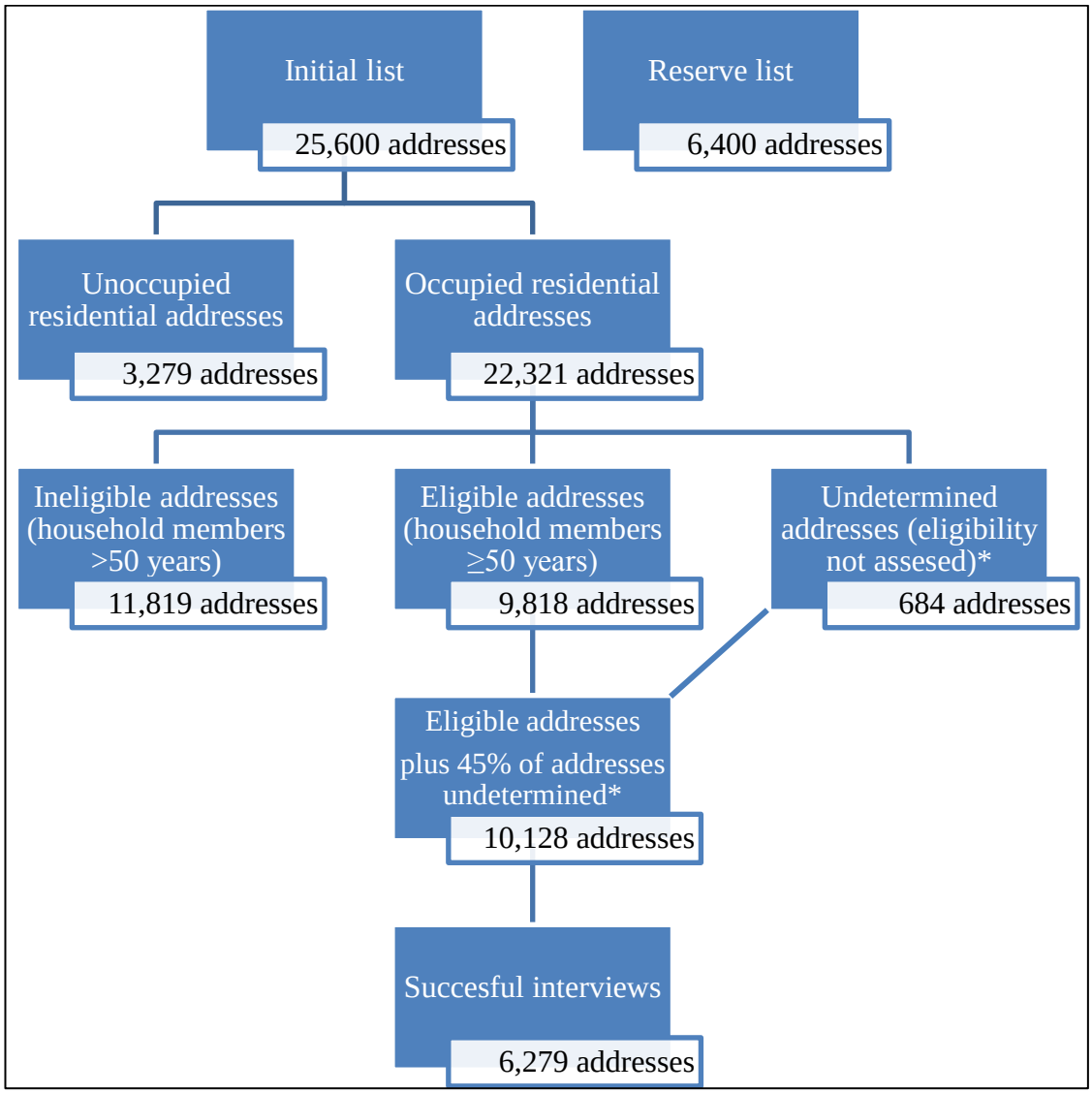
Table 2.2 Summary of health variables collected in wave 1 of the TILDA health assessment.

Variable	Health centre	Home	Number of measurements	Equipment
Height*	✓	✓	One	SECA 240 wall mounted measuring rod
Weight*	✓	✓	One	SECA electronic floor scales or SECA seated scales
Waist size	✓	✓	Two	Standard tape measure
Hip size	✓	✓	Two	Standard tape measure
Blood pressure*	✓	✓	Three- 2 seated and 1 standing	OMRON digital automatic blood pressure monitor
Heart rate	✓	✓	Three	OMRON digital automatic blood pressure monitor
Phasic blood pressure	✓		Six- 1 at baseline, 1 nadir and 4 at 30 second intervals after active stand	Finometer
Pulse wave velocity	✓		Two	Vicorder
Heart rate variability	✓		One 10-minute recording	Medilog Darwin AR12
Visual acuity	✓		Two- Left and right eye	Logmar chart
Contrast Sensitivity	✓		Two- Dim light and without glare	Stereo Optical Co, Functional Visual Analyser
Retinal photograph*	✓		Two- Left and right eye	NIDEX- Non-mydriatic Auto-Fundus Camera
Macular pigment optical density*	✓		Twelve- 6 measurements per eye	Macular Metrics Densitometer
Bone density	✓		One- non dominant foot	Achilles Insight Heel Ultrasound
Venous blood sample*	✓	✓	25 mls	Standard blood taking materials
Depression	✓	✓	One	8-item CES-D scale
Global cognition	✓	✓	Two	1) Cognitive assessment (MOCA) 2) Mini Mental State Examination (MMSE)
Attention	✓	✓	One	Sustained Attention Response Time
Visual memory	✓	✓	One	CAMDEX Picture Memory Test (Acquisition, Free recall, Recognition)
Speed of processing	✓	✓	One	Choice reaction time test and Timed colour trails 1 and 2
Executive function	✓	✓	Three	Visual reasoning - CAMDEX
Grip strength	✓	✓	Four- 2 readings on each hand	Baseline Hydraulic Hand Dynamometer
Timed Up and Go	✓	✓	One	Standard tape measure/chair/tape
Assessment of gait	✓		Three- Normal walk, walk with manual task, walk with cognitive talk	GAITRite Sensored Mat

* Variables of interest in this thesis; comprehensive health centre assessment carried out by a team of trained nurses in one of two dedicated health centres (located in Dublin and Cork), if the participant was unable/unwilling to travel, a modified assessment was carried out in the participant's own home.

2.1.10 Response rate

The overall response rate for wave 1 of TILDA was 62% of selected households (6,279 of 10,128 households that had a successful interview from an eligible participant) (see Figure 2.3).



*Based on those households in which eligibility was determined, it is estimated that 45% undetermined households [9818/ (9818+11819) x 684 = 310.4] were eligible. The estimated number of selected eligible households is therefore 9818 + 310.4 = 10128.4.

Figure 2.3: Response rate of the selected households in wave 1 of TILDA.

In total, for wave 1 (baseline data), 8,175 participants aged 50 years and over from 6,279 households participated in the TILDA study. Of these participants, 5,897 participants (72%) completed a health assessment (at one of the dedicated health centres [n=5,036] or modified home assessment [n=861]).

2.1.11 Strengths and weakness of the study

The large number of variables collected by the TILDA study was a strength in itself, but also the sampling method that provided a nationally representative sample was the primary strength of the study. This cohort also represents a racially homogenous sample (99% were white and Irish born). The design of TILDA was extensively tested and refined by two pilot studies.[5] Although, TILDA is a longitudinal study, the data collected as part of wave 1 data must be treated as cross-sectional. TILDA was able to measure the prevalence of disease such as AMD and other factors, for the first time in many cases, in Ireland.[9] This provided the opportunity to study multiple outcomes and exposures that are important for public health and the assessment of the burden of disease.

However, TILDA was susceptible to bias due to a lower response in those aged 75 years and older and the exclusion of residents in nursing homes or other institutions compared to the overall population aged over 50 years in Ireland. Although the inclusion of the home-based health assessment was to limit the under-representation of older and frailer individuals, retinal photographs and MP measurements were not obtained unless the participant attended the health centre. Particularly for the interest of

this thesis, the lack of dietary data in wave 1 limited the interpretation of the role of nutrients on health outcomes.

2.2 Plasma carotenoid analysis by high performance liquid chromatography

2.2.1 Principle

Analytical chemistry consists of methods to separate, identify and quantify analytes of interest from a mixture (e.g. plasma sample). HPLC is an analytical technique by which a sample is separated by its partition between two phases. The principle of HPLC is that liquid (mobile phase) is pumped through a column of porous material (stationary phase). A sample is injected onto a column and the separation of analytes are based on their physiochemical interactions (i.e. migration rate) between the stationary and mobile phases. The rate it takes for the analyte to elute from the column depends on the partition behaviour of the analytes (e.g. polarity). Each analyte elutes from the column in a particular order depending on the strength of its interaction between the two phases, which is also dependent on the mode of HPLC (normal-phase or reversed-phase).

Carotenoid separation can be performed by both reversed-phase and normal-phase HPLC. The reversed-phase method uses a relatively polar mobile phase and a nonpolar (hydrophobic) stationary phase, which has been used throughout laboratories around the world.[10-13] During reversed phase separation, polar carotenoids (xanthophylls) such as L and total Z partition more effectively into the mobile phase and, therefore, elute first while nonpolar carotenoids (carotenes) such as beta-carotene

and lycopene are retained on the column longer. The normal-phase separation allows further quantification and qualification of xanthophylls and their enantiomers (e.g. Z and MZ).[14] In summary, HPLC is a powerful technique, which is used for separation, identification and quantification of carotenoids in human plasma and tissue.

2.2.2 Instrumentation

The HPLC system used in this study was an Agilent 1260 Series consisting of a quaternary pump, autosampler, thermostat column compartment, fraction collector and a photodiode array detector monitoring a wavelength of 450 nm for plasma carotenoids and 292 nm for tocopherols, as shown in Figure 2.4.

Figure 2.4: Instrumentation of a high performance liquid chromatography (HPLC).

2.2.3 Standards and solvents

The L and Z standards were purchased from CaroteneNature (Lupsingen, Switzerland). All other standards were purchased from Sigma-Aldrich (Arklow, Ireland). HPLC grade

solvents used for extraction and HPLC analysis were supplied by Sigma-Aldrich (Arklow, Ireland), Fisher Scientific (Dublin, Ireland), and VWR (Dublin, Ireland).

2.2.4 Method development

Before the initiation of this study, the Macular Pigment Research Group (MPRG) biochemistry lab operated on two successive methods for liquid chromatography separation of L, MZ and Z from human serum/plasma.[14] Assay 1 (reversed-phase) separated L and total Z (a combined peak of Z and MZ), where the combined peak was automatically collected using a fraction collector for separate analysis (Assay 2). "Total" refers to the stereoisomers (same molecular formula as another molecule, but with a different arrangement of the atoms in space) (see section 1.5.5 of Chapter 1). Assay 2 (normal-phase) individually quantified Z and MZ. In normal-phase HPLC, the mobile phase was non-polar (hexane and isopropanol) and the stationary phase was a Chiral column, which separated stereoisomers (i.e. enantiomers).[14]

The HPLC method development involved for this study focused L and total Z using Assay 1 (reversed-phase HPLC), which used a C18 column and a premixed isocratic mobile phase (acetonitrile, methanol and triethlyamine) and dichloromethane (see Table 2.3).

Method development was accomplished using a standard mixture of alpha-tocopherol acetate (synthetic vitamin E as internal standard [IS]) and 6 carotenoids (L, Z, beta-cryptoxanthin, alpha carotene, beta-carotene, and lycopene) and volunteer plasma samples.

Table 2.3: HPLC parameters for the analysis of plasma samples using a C18 column.

HPLC parameters of Assay 1 (MPRG method)	
Column	Phenomenex Ultracarb 3µ C18 column (250 x 4.6 mm)
Column Temp.	15°C
Detection	450 nm and 292 nm
Flow rate	0-15 min (1 ml/min); 15-27 min (2 ml/min); 27-34 (1 ml/min)
Injection volume	100 µl
Mobile phase A	85% acetonitrile, 15% methanol and 0.1% triethlyamine
Mobile phase B	Dichloromethane
Binary gradient	0-15min (to 0% B); 15-16 min (10% B); 25-27min (to 50% B); 27-34 min (0% B)
Run time	34 minutes

High performance liquid chromatography (HPLC); Macular Pigment Research Group (MPRG).

L and total Z peaks eluted at approximately 12.7 and 14 mins, respectively, and a high gradient of dichloromethane co-eluted all remaining carotenoids (Figure 2.5). These parameters allowed for the accurate quantification and assessment of L and total Z.

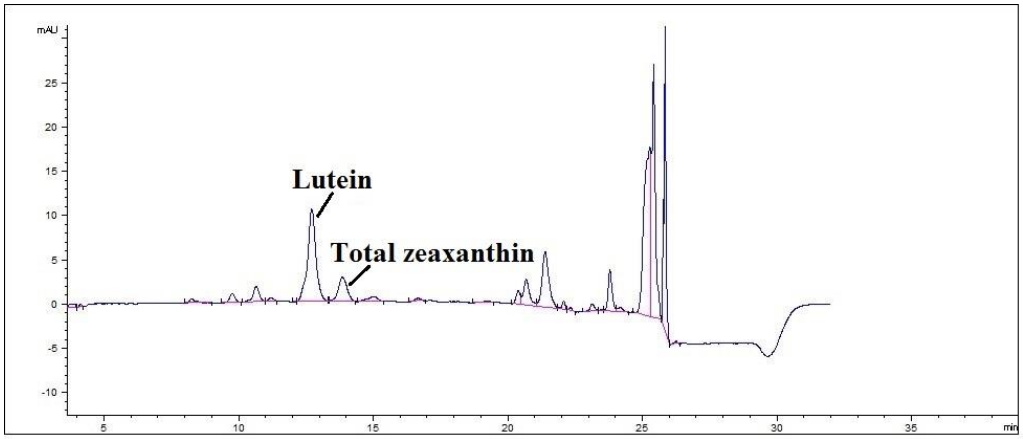


Figure 2.5: HPLC chromatograph of a plasma sample using a C18 column (250 x 4.6 mm, 3 µm); Total zeaxanthin refers to the co-eluted zeaxanthin and meso-zeaxanthin; mAU = milli-absorbance units; min= time in minutes.

However, the co-eluted carotenoids (potentially lycopene, beta-cryptoxanthin alpha and beta carotene) presented in this chromatograph (Figure 2.5) were also of interest (particularly in a large population study). For example, alpha and beta carotene (linked to pro-vitamin A activity),[15] lycopene (linked to prostate cancer),[16] and beta-cryptoxanthin (linked to arthritis).[17] Therefore, method development to identify and quantify all the major plasma carotenoids was undertaken for this study. The most common parameters that were changed during method development are briefly discussed below.

Mobile phase

The gradient of the mobile phase was altered to change the interactions of the carotenoids of interest between the two phases. As we know from the previous HPLC parameters mentioned in Table 2.3, dichloromethane was useful for rapidly eluting carotenoids. Therefore, instead of using 50% of dichloromethane, a step-gradient approach using a smaller amount (10%) was used before returning to the initial conditions (Table 2.4). As a less abrasive gradient was used, the run time was increased to allow the carotenoids time to elute.

Column temperature

Column temperature is an important factor that can effect separation, resolution and reproducibility of retention times of carotenoids. Bohm *et al*. tested the column temperatures between 15°C and 25°C and found 23°C was the best compromise for the separation of the major carotenoids.[18] After considering this change, the column temperature was increased from 15°C to 20°C to try improve the carotenoid separation.

Table 2.4: HPLC parameters for the analysis of plasma samples using a different mobile gradient.

HPLC parameters of Assay 1	
Column	Phenomenex Ultracarb 3µ C18 column (250 x 4.6 mm)
Column Temp.	20°C
Detection	450 nm and 292 nm
Flow rate	1 ml/min
Injection volume	100 µl
Mobile phase A	85% acetonitrile, 15% methanol and 0.1% triethlyamine
Mobile phase B	Dichloromethane
Binary gradient	0-15min (to 10% B); 15-30min (10% B); 30-35min (to 0% B); 35-45min (0% B)
Run time	45 minutes

L and total Z peaks eluted at approximately 11 and 11.7 mins, respectively, followed by beta-cryptoxanthin, lycopene, alpha-carotene and beta-carotene (Figure 2.6). However, the carotenoid separation was not ideal as it was obvious there was peak tailing (asymmetrical peaks) after 18 mins. Upon examination of the ultraviolet–visible (UV-Vis) absorption spectra of each carotenoid of interest, it was noted there was a *cis* peak co-eluting with the total Z peak. All other carotenoids of interests were all-*trans* form. The occurrence of this *cis*-carotenoid was identified by the presence of an additional spectral peak at 332 nm and a hypsochromic shift of 12 nm (to shorter wavelengths) comparted to the UV spectra of a Z standard. Of note, it has proven difficult to separate such *cis*-carotenoids using a C18 column as the column needs to recognise and resolve subtle molecule differences.

A)

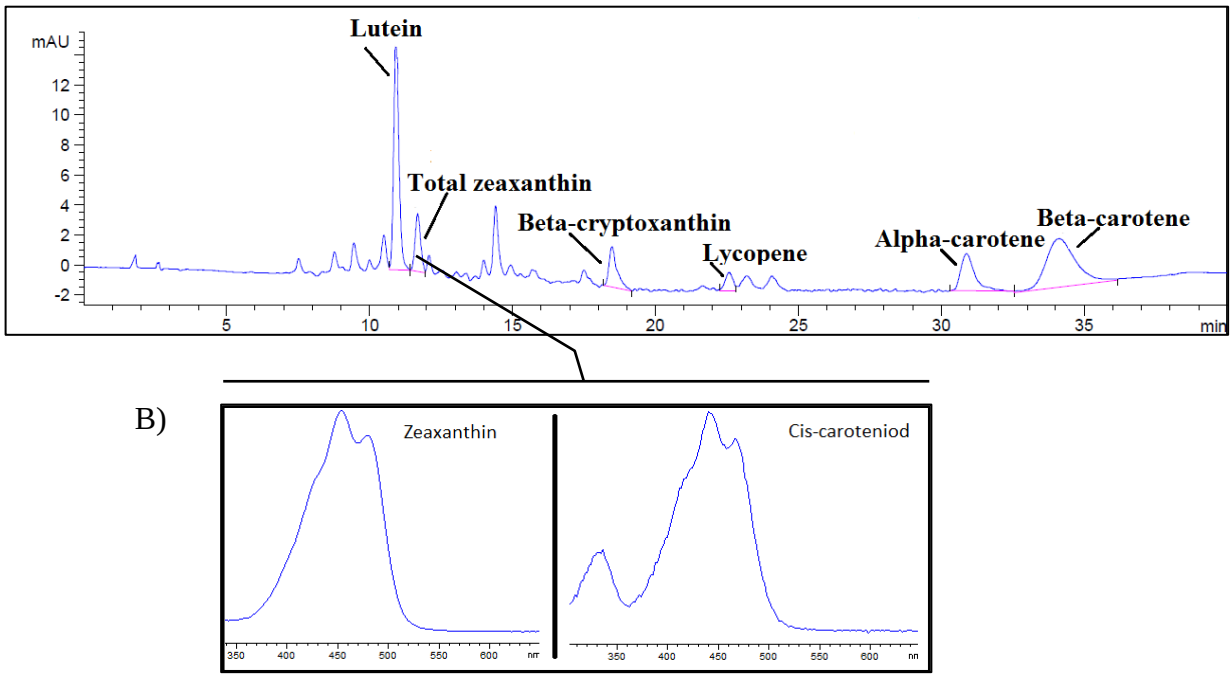


Figure 2.6: A) HPLC chromatograph of a plasma sample using a C18 column (250 x 4.6 mm, 3 µm) and a different mobile gradient; Total zeaxanthin refers to the co-eluted zeaxanthin and meso-zeaxanthin; mAU = milli-absorbance units; mi = time in minutes; B) Ultraviolet–visible absorption spectra of total zeaxanthin peak.

Analytical column

Based on the review of literature,[11, 19-21] the column in Assay 1 was changed to the YMC C30 column (250 x 4.6 mm, 3 µm) with a 10 x 4 mm, 3 µm YMC Guard Cartridge (guard and column, Apex Scientific, Kildare, Ireland), which was more hydrophobic when compared to the C18 column. This enabled the resolution of polar and nonpolar geometric carotenoids isomers. Successful application of this column has been used in the analysis of blood samples, foods, algae, and natural extracts.[22-24] Using a modified method from Yeum *et al.*,[11] a good separation was achieved using methanol, methyl tert-butyl ether (MTBE) and water (only a low content of water was used to avoid loss in selectivity) using the parameters as shown in Table 2.5.

Butylated hydroxytoluene (BHT) was added to both mobile phases to reduce carotenoid degradation during HPLC analysis.[25] The column temperature was kept low (16°C) to maximise selectivity for *trans/cis* isomers.[26] The HPLC gradient eluted all carotenoids of interest in 38-minutes.

Table 2.5: HPLC parameters for the analysis of plasma samples using a YMC C30 column.

HPLC conditions of Assay 1	
Column	YMC Carotenoid 3µ C30 column (250 x 4.6 mm)
Column Temp.	16°C
Detection	450 nm and 292 nm
Flow rate	1 ml/min
Injection volume	100 µl
Mobile phase A	83% Methanol, 15% MTBE, 2% Water and 0.1% butylated hydroxytoluene (BHT)
Mobile phase B	90% MTBE, 8% Methanol, 2% Water and 0.1% BHT
Binary gradient	Initially 5% B, 0-12 min (to 20% B); 12-20 min (55% B); 20-27min (to 95% B); 27-30 min (95% B), followed by resuming to initial setting at 33 min
Run time	38 minutes

L and total Z peaks eluted at approximately 8 and 9.4 mins, respectively, followed by beta-cryptoxanthin, alpha-carotene, beta-carotene and lycopene (Figure 2.7). The YMC C30 column was also capable of separating *trans* and *cis* carotenoids. The configuration of *cis*-carotenoids can be confirmed by a known standard of *cis*-isomer of that carotenoids, however this is difficult to source and costly to produce.

A)

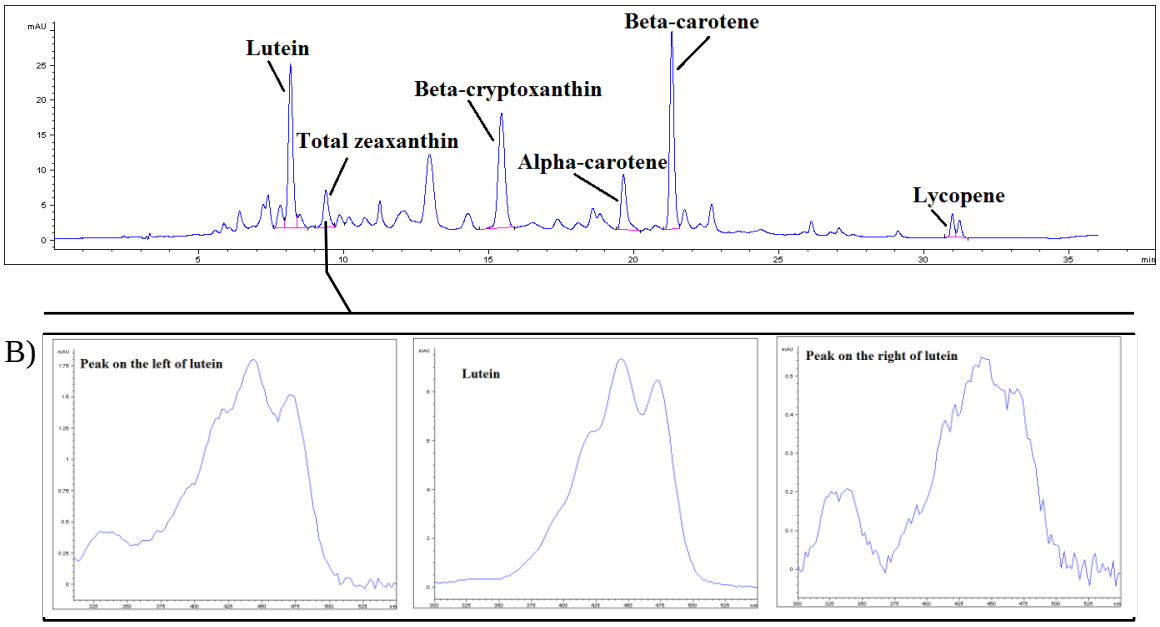


Figure 2.7: A) HPLC chromatograph of a plasma sample using a YMC C30 column (250 x 4.6 mm, 3 µm); Total zeaxanthin refers to the co-eluted zeaxanthin and meso-zeaxanthin; mAU = milli-absorbance units; min = time in minutes B) ultraviolet–visible absorption spectra of lutein peak and two peaks on either side of lutein.

In summary, the application of the YMC C30 column slightly reduced the run time (compared to the parameters in Table 2.4), which was a desirable when analysing a large population size (TILDA, n=5,000) and improved quality of carotenoids analysis, including their *cis*-isomers.

2.2.5 Quantitation of plasma carotenoids

Plasma L and total Z concentrations (Z and MZ) were quantified using the response factors for the carotenoid of interest reported in this section, which are specific to the

C30 column used and the injection volume. Carotenoid concentrations were calculated as μmol/L.

$$Plasma\ caroteniod\ conc.\ (\mu mol/l) = RF\ x\ \left(\frac{100\%\ IS}{Acutal\ IS}\right) x\ PA\ x\ DF$$

Equation 2.1: calculation used to quantity plasma carotenoids. Where RF: response factor; 100% IS: peak area taken from IS only chromatogram; Actual IS: Internal standard peak area taken from sample chromatogram; PA: Peak Area; DF: Dilution Factor = 0.5 (0.4 ml of plasma was used for extraction with a final volume of 0.2 ml reconstituted sample for injection).

$$Response\ factor\ (RF) = \frac{concentration\ of\ injected\ standard}{PA}$$

Equation 2.2: calculation used to determine response factor (RF). Where the concentration of injected is concentration of standard; PA: Peak Area.

2.2.6 Method Validation

A full report on the method validation of L and Z using YMC C30 columns (Part No. 0425053733 and 0425060305) and the performance of the HPLC are discussed below. Due to the volume of samples ran on the first YMC C30 column (Part No. 0425053733), this column was retired and a new YMC C30 Column (Part No. 0425060305) fitted to the HPLC. During the ten months of HPLC analysis, performance reports were carried out every three months or following the replacement of major HPLC parts (e.g. UV-Vis lamp or column). A standard mixture of carotenoids (L, Z, beta-cryptoxanthin, alpha-carotene, beta-carotene and lycopene) plus alpha-tocopherol was run every day to check retention times and precision was measured weekly.

Precision

Precision is defined as the degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of a homogenous sample. The relative standard deviation (RSD) is a measure of precision, calculated by dividing the standard deviation (SD) for a series of measurements by an average measurement:

$$RSD\% = \frac{100\%\ SD}{Average\ measurement}$$

- A standard L solution was injected three times and the RSD% calculated
- A standard Z solution was injected three times and the RSD% calculated
- Specification was RSD% ≤ 2%.

Table 2.6: Results of precision test for lutein and zeaxanthin using a YMC C30 column (Part No. 0425053733).

Injections	**Lutein (peak area)**	**Zeaxanthin (peak area)**
1	479.5	158.4
2	477.4	157.0
3	476.4	155.8
Average	477.77	157.06
Standard deviation	1.58	1.30
RSD (%)	**0.33**	**0.82**

Table 2.7: Results of precision test for lutein and zeaxanthin using a YMC C30 column (Part No. 0425060305).

Injections	**Lutein (peak area)**	**Zeaxanthin (peak area)**
1	244.9	44.6
2	245.7	44.0
3	244.5	43.8
Average	245.03	44.13
Standard deviation	0.611	0.410
RSD (%)	**0.2449**	**0.9434**

In summary, the RSD values obtained in both columns for L and Z were within the required specification and therefore the assay has passed the precision test for the compounds of interest (i.e. L and Z).

Accuracy

The accuracy of a measurement is defined as the closeness of the measured value to the true value. In a method with high accuracy, a sample (whose "true value" is known) is analysed. The measured value should be comparable to the true value. The true value was the Standard reference material (SRM): SRM 968e - Fat-Soluble Vitamins, Carotenoids, and Cholesterol in Human Serum, provided by the National Institute of Standards and Technology (NIST) (Appendix C).

$$Plasma\ caroteniod\ conc.\ (\mu mol/l) = RF\ x\ \left(\frac{100\%\ IS}{Acutal\ IS}\right) x\ PA\ x\ DF$$

NIST Standard Level 2.1 (external standard) for YMC C30 column (Part No. 0425053733)

100% Internal standard (IS) = 1409.48

Actual IS: 1002.3

DF: 0.5

Response Factor (RF) (Part No. 0425053733):

RF for Lutein	0.001756
RF for Zeaxanthin	0.002316

Lutein value reported by NIST certificate for SRM:

0.170 ± 0.013 µmol/mL (0.157-0.183 µmol/mL)

Lutein PA= 143.3

Concentration of Lutein in sample = 0.001756 x (1409.48/1002.3) x 143.3 x 0.5

Concentration of Lutein in sample **= 0.1769 µg/L (passed)**

Zeaxanthin value reported by NIST certificate for SRM:

0.052 ± 0.010 µmol/L (0.042-0.062 µmol/L)

Zeaxanthin PA= 33.8

Concentration of Zeaxanthin in sample = 0.002316 x (1409.48/1002.3) x 33.8 x 0.5

Concentration of Zeaxanthin in sample = **0.055 µg/L (passed)**

NIST Standard Level 3.2 (external standard) for YMC C30 column (Part No. 0425060305)

100% IS = 2237.46

Actual IS: 1614.58

DF: 0.5

Response Factor (RF) (Part No. 0425060305):

RF for Lutein	0.001816
RF for Zeaxanthin	0.003270

Lutein value reported by NIST certificate for SRM: 0.218 ± 0.017 µmol/mL (0.201-0.235 µmol/mL)

Lutein PA= 183.57

Concentration of Lutein in sample = 0.001816 x (2237.46/1614.58) x 183.57 x 0.5

Concentration of Lutein in sample **= 0.231 µg/L (passed)**

Zeaxanthin value reported by NIST certificate for SRM: 0.052 ± 0.009 µmol/L (0.042-0.062 µmol/L)

Zeaxanthin PA= 26.6

Concentration of Zeaxanthin in sample = 0.003270 x (2237.46/1614.58) x 26.6 x 0.5

Concentration of Zeaxanthin in sample = **0.060 µg/L (passed)**

Selectivity

Selectivity is the ability to find and quantify the compound of interest in the presence of other compounds. For chromatographic methods, this means that the analyte of interest can be separated with sufficient resolution from all other peaks.

- A combined standard solution consisting of L and Z was run three times on the HPLC system.
- The specification: The average resolution between these two analytes (calculated using Chemstation software) was required to be ≥ 1.5.

Table 2.8**:** Results of selectivity test for lutein and zeaxanthin using a YMC C30 column (Part No. 0425053733).

Injections	**Resolution L/Z**
1	5.29
2	5.30
3	5.33
Average	5.31

Table 2.9: Results of selectivity test for lutein and zeaxanthin using a YMC C30 column (Part No. 0425060305).

Injections	**Resolution L/Z**
1	5.72
2	5.69
3	5.67
Average	5.69

The average resolution between L and Z was within the required specification and therefore has passed the selectivity test.

Limited of detection and quantification

Detection is the ability to analyse samples with low content. The limit of detection (LOD) can be defined as the smallest level of analyte that gives a measurable response (i.e. analyte concentration that gives a peak signal 3.3 times the baseline noise). The limit of quantification (LOQ) can be defined as the smallest concentration of analyte which gives a response that can be accurately quantified (i.e. analyte concentration that gives a peak signal 10 times the baseline noise).

$$LOD: 3.3\ x\ S/N$$

$$LOQ: 10\ x\ S/N$$

$$S/N = (2H/h)$$

$$S/N = Signal \cdot to \cdot noise\ ratio$$

$$H = height\ of\ peak$$

$$h = height\ of\ baseline\ noise$$

Table 2.10: Results of precision test for lutein and zeaxanthin using a YMC C30 column (Part No. 0425053733).

Limit of detection		Limit of quantification	
Lutein:	0.0005 µmol/L	Lutein:	0.0015 µmol/L
Zeaxanthin:	0.0008 µmol/L	Zeaxanthin:	0.0023 µmol/L

Table 2.11: Results of precision test for lutein and zeaxanthin using a YMC C30 column (Part No. 0425060305).

Limit of detection		Limit of quantification	
Lutein:	0.0004 µmol/L	Lutein:	0.0008 µmol/L
Zeaxanthin:	0.0011 µmol/L	Zeaxanthin:	0.0025 µmol/L

In summary, the assay displays adequate sensitivity for L and Z.

Linearity and range

A standard curve is a graph of peak area (detector response) versus analyte concentration. A straight line (specification: $R^2 = 1 \pm 0.005$) should be achieved. From the equation of the line (area= slope [analyte concentration] + intercept), unknown analytes can be determined. Examples of standard curves produced for L are shown in Figures 2.8 and 2.9 and standard curves produced for Z are shown in Figures 2.10 and 2.11.

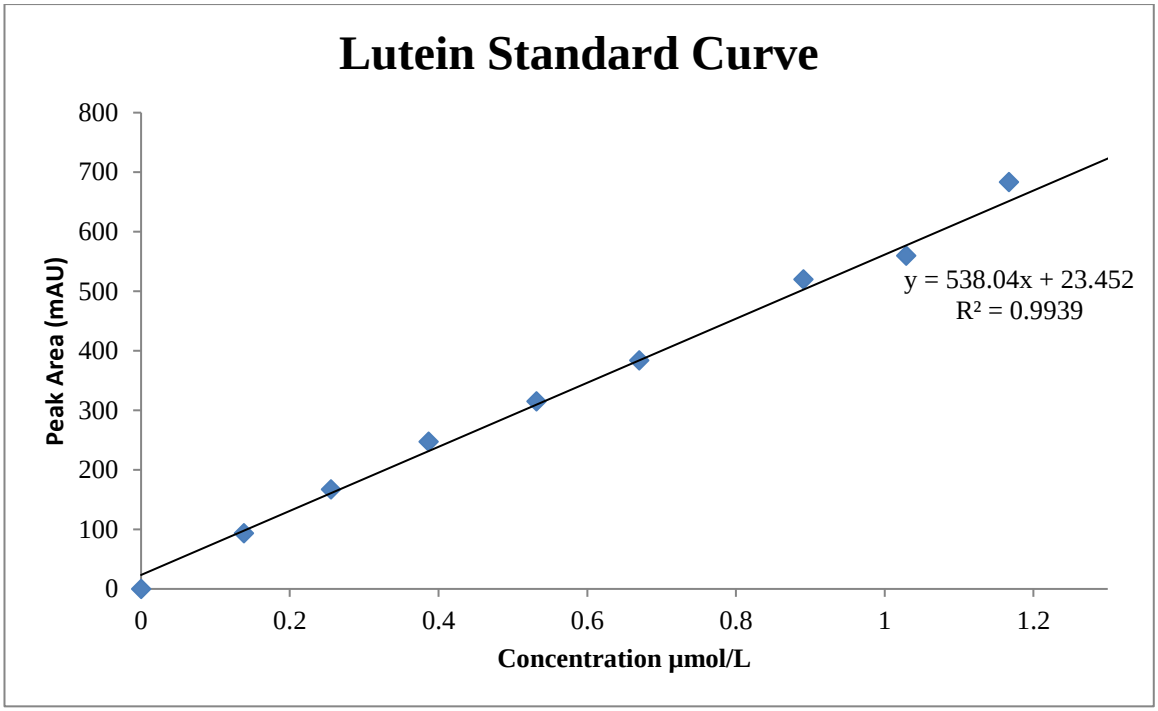


Figure 2.8: Lutein standard curve used to quantify plasma concentrations (YMC C30 column [Part No. 0425053733]).

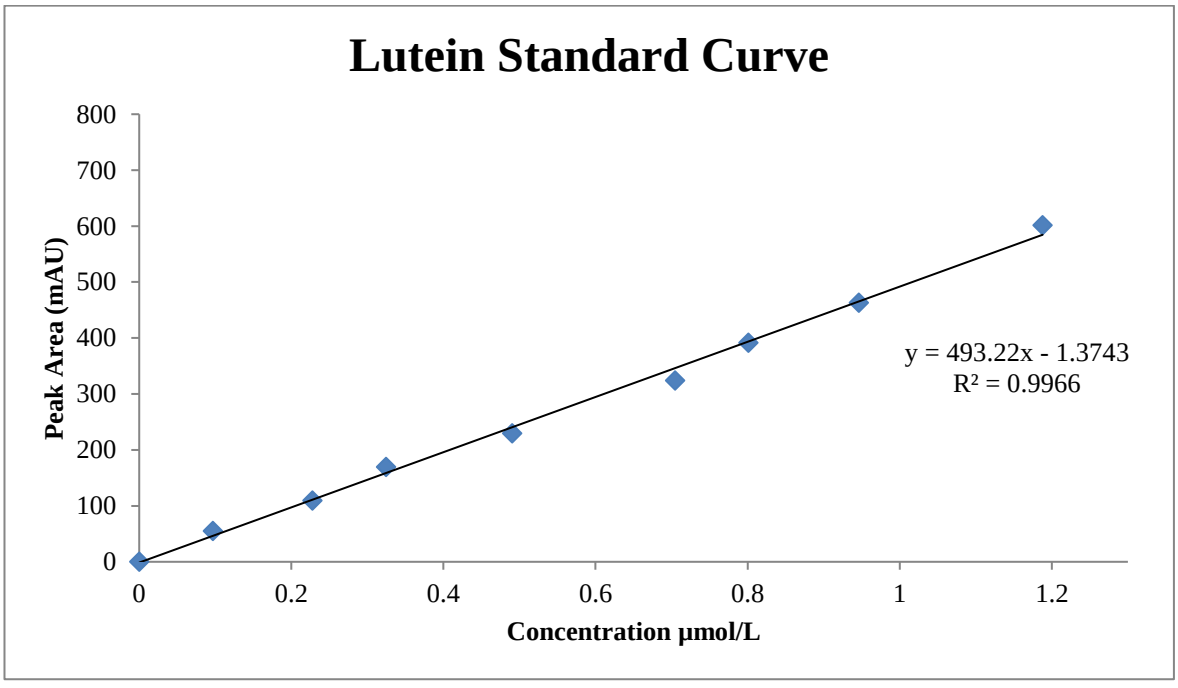


Figure 2.9: Lutein standard curve used to quantify plasma concentrations (YMC C30 column [Part No. 0425060305]).

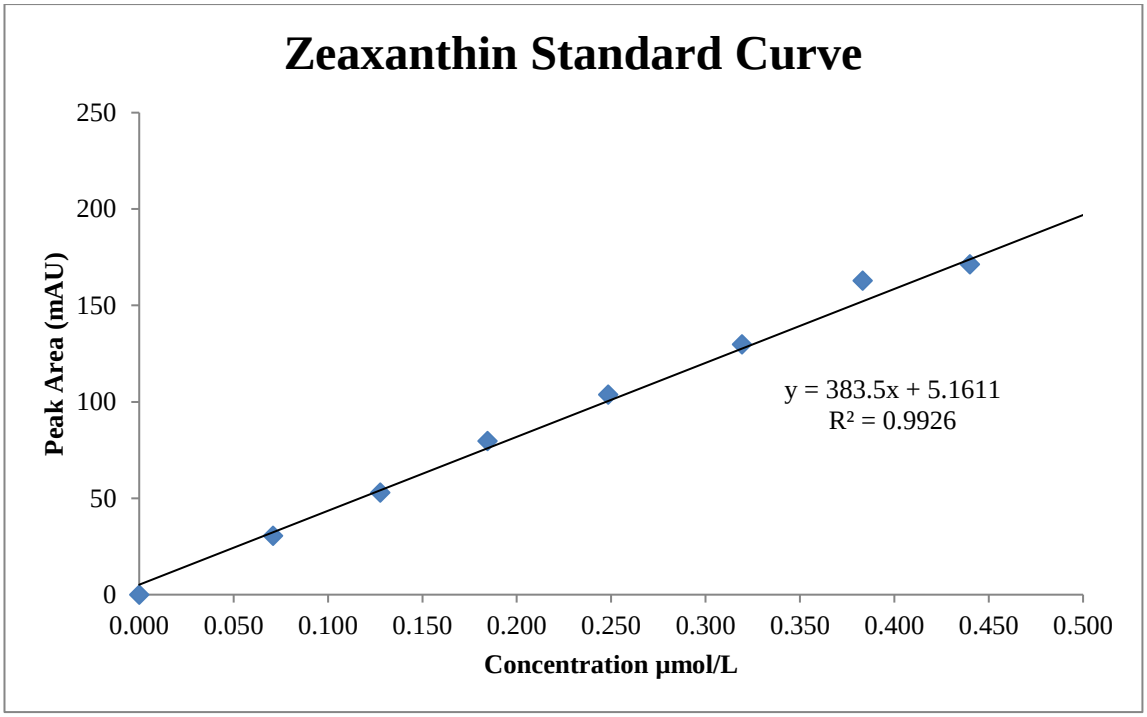


Figure 2.10: Zeaxanthin standard curve used to quantify plasma concentrations (YMC C30 column [Part No. 0425053733]).

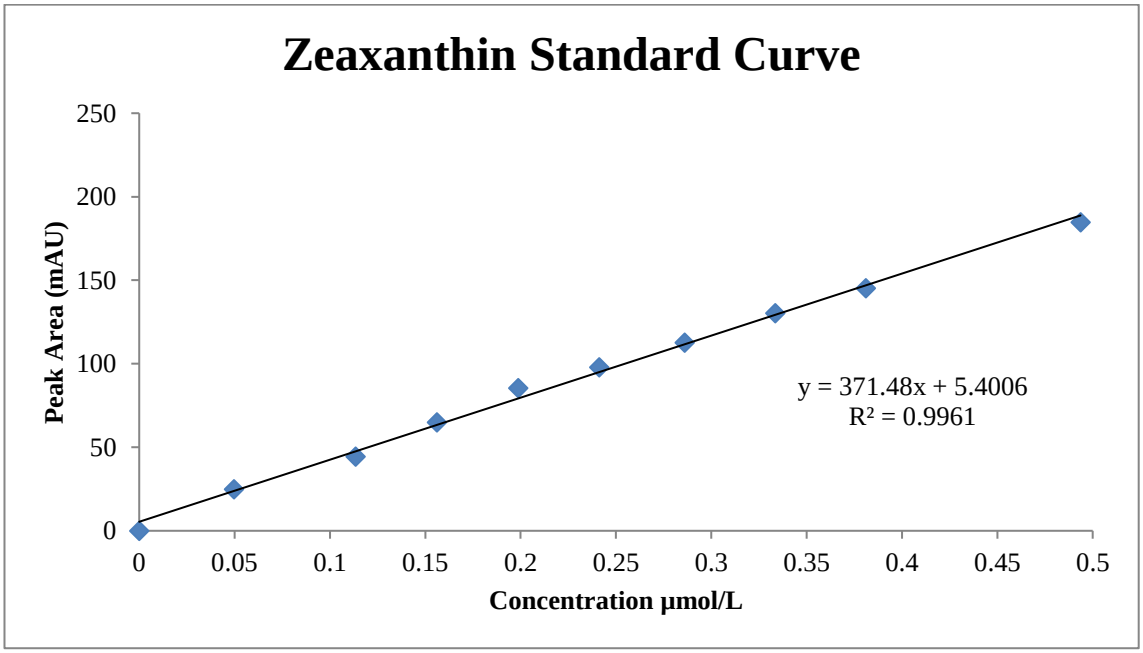


Figure 2.11: Zeaxanthin standard curve used to quantify plasma concentrations (YMC C30 column [Part No. 0425060305]).

Quality control

As the NIST standard was analysed at the beginning and end of project, control samples (plasma from the same individual) were analysed at least once a month to continue monitoring the accuracy of this method. Average coefficients of variation were 4% and 6.8% intra-assay for L and Z, respectively (Table 2.12). Intra-assay precision was ≤10%.

Table 2.12: Plasma concentrations of control plasma samples over 10 months.

Sample ID	**Lutein (µmol/L)**	**Zeaxanthin (µmol/L)**
JC1.1	0.522	0.293
JC1.2	0.523	0.287
JC1.3	0.520	0.297
JC1.4	0.526	0.303
JC1.5	0.524	0.311
JC1.6	0.517	0.314
JC1.7	0.530	0.317
JC1.8	0.551	0.357
JC1.9	0.583	0.343
JC1.10	0.504	0.349
JC1.11	0.544	0.343
JC1.12	0.546	0.340
JC1.13	0.549	0.320
JC1.14	0.564	0.363
JC1.15	0.587	0.349
JC1.16	0.551	0.349
JC1.17	0.549	0.326
JC1.18	0.545	0.337
JC1.19	0.549	0.336
Average	0.543	0.329
Standard Deviation	0.02184	0.02246
Average coefficients of variation	**4%**	**6.8%**

2.3 Plasma carotenoid extraction method

The following plasma carotenoid extraction method was used for all TILDA plasma samples and the method was sourced from a report by Connolly *et al.* (Figure 2.12).[27]

Over 5,000 plasma samples were transported from the laboratory in Trinity College Dublin on dry ice by a pathology storage company (Advance Diagnostic Ltd Products, Wicklow, Ireland). Of note, each sample was wrapped in tinfoil and stored in black cyroboxes (81 samples per box). Once the samples were received at MPRG (Waterford Institute of Technology, West Campus, Waterford, Ireland), TILDA identifications (TILDA ID) were recorded, a photograph of each box was taken and samples were stored at -80˚C until time of extraction. TILDA IDs were cross-checked with a TILDA research fellow and TILDA ID Eppendorf labels were printed for each sample. Samples were defrosted in batches of 18 for carotenoid extraction.

Plasma (0.4 mL) was micropipetted into clear 1.5 mL Eppendorf tubes labelled according to unique TILDA ID. IS ([0.2 mL α-tocopherol acetate] [250 mg/L ethanol]), 0.3 mL of BHT (250 mg/L ethanol) and 0.5 mL of heptane were added. The mixture was vortexed using a Vortex Genie-2 (Scientific Industries, Dublin, Ireland) at the highest setting for 2 minutes, followed by centrifugation with an AccuSpin Micro 17 (Fisher Scientific Ireland, Dublin, Ireland) for 5 minutes at 400 g.

An aliquot of the upper heptane layer (0.4 mL) was removed to a light resistant (amber) Eppendorf tube, and the heptane extraction was repeated once more, adding a further 0.5 mL of heptane to the original residue. The combined extracts were dried under nitrogen and stored at -80˚C until analysis.

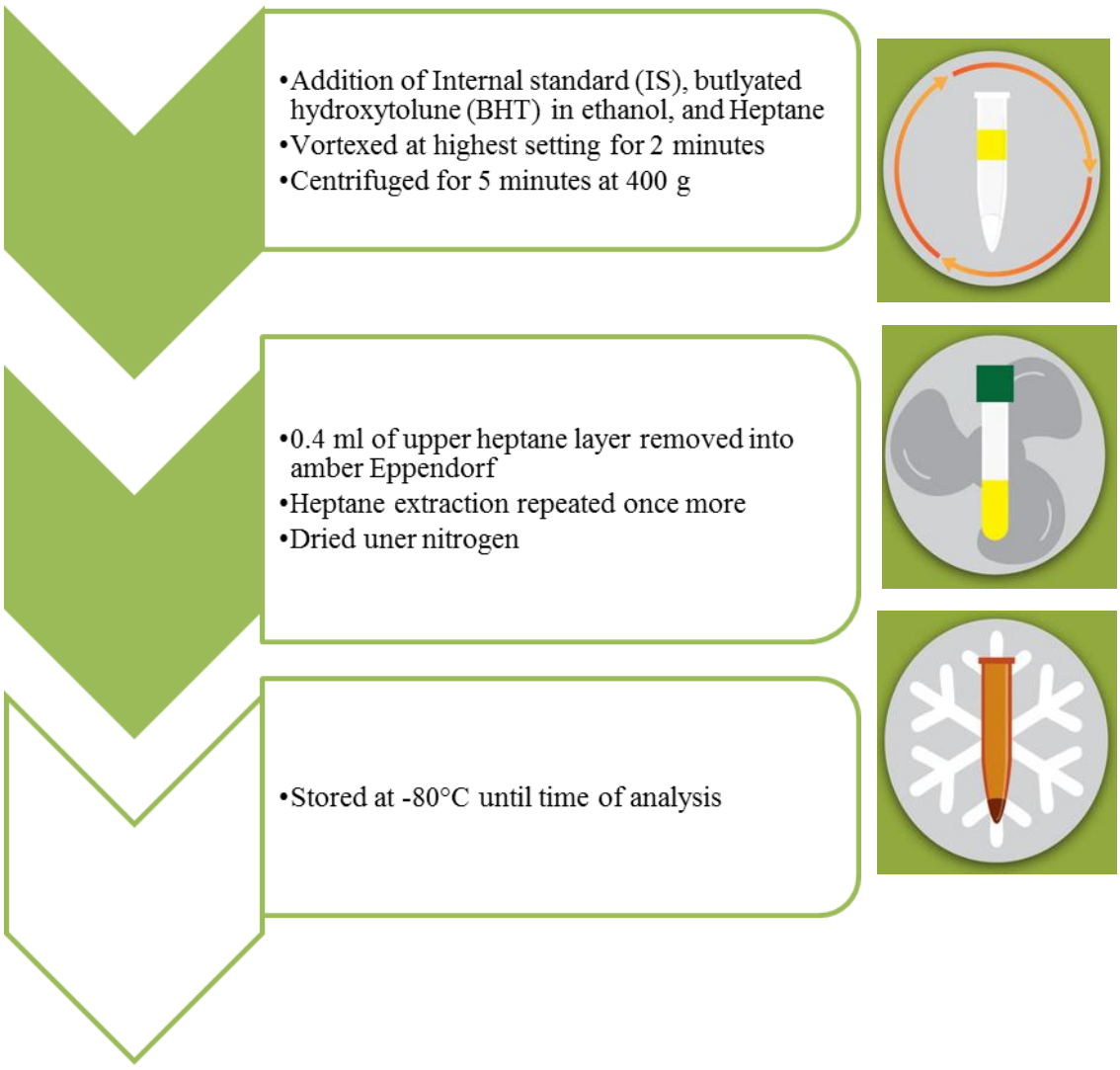


Figure 2.12: Carotenoid extraction from human plasma.

2.4 Data management

All plasma concentrations data for L and Z were entered into an excel spreadsheet by the main investigator (RM), and cross-checked to a hard copy chromatograph print out for each sample. Random checks were made (summer students), to ensure that all data was entered correctly. The data was transferred in an encrypted format and saved on to a USB, which was hand delivered to the data manager in TILDA for data merging. A TILDA postdoctoral fellow carried out cross-checks on the data to ensure the dataset was harmonised.

The statistical package IBM SPSS Statistics for Windows Version 22.0 was used for analysis. General linear models and logistic regression were used to assess the relationship between a dependent variable (plasma L and Z and AMD, respectively) and independent variables. Full details of the statistical analysis employed are described in Sections 3.2.8 and 4.2.7. The 5% level of significance was used throughout, without adjustment for multiple testing.

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Chapter 3: Non-dietary correlates and determinants of plasma lutein and zeaxanthin concentrations in the Irish Population

3.1 Introduction

Three carotenoids, L, Z and MZ, accumulate in the macula, where they are collectively referred to as MP.[1, 2] The macula, a specialised area of the retina, is responsible for central and colour vision. The blue light-filtering[3] and antioxidant properties[4] of MP render this pigment important for optimising visual function in humans. Indeed, MP has been shown to enhance visual function in diseased[5, 6] and non-diseased eyes[7] and reduces the risk of visual loss in, and progression of, AMD,[8] the leading cause of blindness in adults over the age of 65.[9-12] Also, several epidemiological studies have shown that participants with a high dietary intake of carotenoids (including L and Z) have a lower prevalence of AMD, when compared to participants with a poor dietary intake of carotenoids.[13-15]

Recent studies have demonstrated that L and Z are found in the monkey and human brain,[16-18] and work by Johnson *et al.* has shown that brain concentrations of these carotenoids correlate positively with MP levels.[19] Also, various groups have reported that MP (and serum concentrations of L and Z) correlates positively to measures of global cognitive function,[20-22] and work from our research group has shown that MP (and serum concentrations of L and Z) is significantly lower in patients with AD, when compared with age-matched controls.[23] Accordingly, there is a need to

understand factors that influence the circulating concentrations of these nutrients deemed important for eye and brain health.

Carotenoids are entirely of dietary origin and, as a result, the plasma concentrations of these compounds are dependent on an individual's dietary intake of food containing these nutrients (e.g. leafy greens, vegetables, fruits and eggs).[24] We know that the concentrations of L and Z in human plasma varies dramatically between individuals.[25] We also know that supplementation with the macular carotenoids (L, Z, and MZ) increases serum concentrations of each respective carotenoid and MP, [26, 27] in different population groups (e.g. participants free of retinal disease,[7] participants with AMD,[6] and participants with AD[28]). However, it has been shown that many other variables (both modifiable and non-modifiable) are likely to influence the concentrations of L and Z in human plasma and host tissues (e.g. retina and adipose tissue).[29] Previous studies suggest that variables such as sex, age, BMI, alcohol consumption, smoking status, physical exercise, serum lipids, genetic background, and ethnicity are associated with plasma concentrations of L and Z.[30-38] However, not all reports are in agreement, probably due to differences in methodologies and populations studied.

In the current chapter, we present findings from TILDA. Baseline data on the social, economic, and health status of 8,175 participants aged 50 years and older was collected between 2009 and 2011 from a random sample in ROI.[39] Given the putative and proven health and functional benefits of L and Z for eye and brain, we hypothesised that it was important to study correlates and determinants of these nutrients in an ageing

population. Of note, TILDA allowed us to address this research question, using its large and uniquely homogeneous study sample.

3.2 Methods

3.2.1 Study design and sampling

Full details of the design, sampling and methodology of TILDA has been previously reported in Section 2.1.[39] In brief, a nationally representative sample of community dwelling adults was drawn from the Irish Geodirectory, a current and comprehensive record of all residential addresses in the ROI. Addresses were selected by means of RANSAM using a three stage process where all household residents aged 50 years or older were eligible to participate.[40] Wave 1 (baseline) recruitment had an overall response rate of 62% (n=8175). It is planned that these participants will be interviewed every 2 years and the health assessment repeated every 4 years over a ten-year period. As TILDA began in 2009, it has now completed wave 1, 2, 3, and 4. The focus of the current study was baseline (wave 1) only. Participants were required to provide written informed consent prior to participation in the study. This study was approved by the Faculty of Health Sciences Research Ethics Committee of Trinity College Dublin and the local Institute committee at the Waterford Institute of Technology. All experimental procedures adhered to the tenets of the Declaration of Helsinki.

3.2.2 Interview/questionnaire

As part of wave 1, participants completed a CAPI carried out by a trained social interviewer in the participants' home. The questionnaire collected detailed information on many aspects of the participants' lives (demographics, lifestyle and behaviours), socio-economic status, self-reported health (physical and medical history) and medication use. A list of medications taken on a daily basis (coded using Anatomical Therapeutic Chemical),[41] including food supplements (defined according to the Directive 2002/46/EC of the European Parliament and the Council of the European Union, 10 June 2002) was recorded for each participant. The interview was followed by a self-completion questionnaire, which the participant could complete and return via post. Of note, participants were asked whether a doctor had diagnosed them with the following medical conditions: high blood pressure, AMD (including family history of AMD), diabetic maculopathy, diabetic retinopathy, cataracts or glaucoma. It is important to note that self-reported data is susceptible to bias, and this limitation must be taken into account when interpreting results here.

3.2.3 Physical health assessment

Participants were invited to attend a comprehensive health assessment carried out by a team of trained nurses in one of two dedicated health centres in Dublin and Cork or have a modified assessment carried out in their own home.[39] Height and weight were measured with Seca™ (Seca Ltd., Birmingham, UK) using a standardised protocol, and BMI was calculated as weight (kg)/height (m^2). According to their BMI, participants were categorised into normal (<25 kg/m^2), overweight (25.1-29.9 kg/m^2) or obese (≥30

kg/m^2). Assessment of MP optical density and retinal photographs for AMD grading were only conducted on the 5,035 participants who attended the health centre (i.e. excluding participants who had home health assessments).

3.2.4 Assessment of macular pigment optical density

Customized heterochromatic flicker photometry (cHFP), a fast and non-invasive procedure, using Macular Densitometer^{TM} (Macular Metrics Corp., Providence, RI, USA) was used to measure MP optical density at the fovea (0.5° eccentricity) with a reference set at 7° eccentricity (peripheral reference locus). The eye with the best visual acuity was chosen for MP assessment. A full description of the protocol used for the TILDA sample is published elsewhere.[42, 43] In brief, the subject was required to achieve isoluminance between a blue light wavelength (absorbed by MP), and a green reference wavelength light (not absorbed by MP). This psychophysical technique is customized for each subject, by optimising the method, taking into account the subject's age and critical flicker frequency (to allow participants reach their end point in testing with minimal variance).[44]

3.2.5 Retinal photographs and AMD grading

TILDA nurses were trained and certified by experts (from the Ocular Epidemiology Reading Centre at the University of Wisconsin, Madison, USA) to take retinal photographs using the NIDEK AFCE-210 non-mydriatic auto-fundus camera, through a non-dilated pupil. Pupils were not dilated as this could influence the results of other health measurements such as gait assessment, which was carried out after retinal

photographs. Retinal photographs were graded by a trained and certified grader using a modified version of the International Classification and Grading System for AMD under the supervision of Moorfields Eye Hospital, Reading Centre, London, UK.[9]

3.2.6 Blood collection and processing

Separate written and verbal consent was required to obtain blood samples from participants. Non-fasting venous blood samples were collected into one 5 ml Lithium Heparin tube (BD, Becton, Dickinson Limited, Oxford, UK) for immediate analysis and two 10 ml EDTA tubes (BD, Becton, Dickinson Limited, Oxford, UK) tubes for long term storage. Samples were immediately analysed for lipid profile by a commercial laboratory, which included TC, HDL, LDL and triglyceride. One of the EDTA tubes was immediately protected from direct light. This blood sample was centrifuged and 1 ml of EDTA plasma was dedicated to carotenoid assessment and stored at -80°C until time of analysis.

3.2.7 Plasma L and Z assessment

Plasma concentrations of L and Z were measured in non-fasting plasma samples and therefore could be influenced by recent dietary intake of these nutrients. Each participant had 1 ml of frozen EDTA plasma wrapped in tinfoil transported to the Macular Pigment Research Group, Vision Research Centre, Waterford, Ireland (www.mprg.ie/). In 2013, L and total Z was analysed using a reversed phase HPLC method. Details of extraction procedures and HPLC analysis are previously described by our research group.[23] Method validation was carried out using 968e Fat-Soluble

Vitamins, Carotenoids, and Cholesterol in Human Serum Reference Standard from the NIST and quality checks were frequently evaluated using control plasma samples. Average coefficients of variation were 4% and 6.8% intra-assay for L and Z, respectively. The limits of quantification were determined to be 0.0021 μmol/L and 0.0027 μmol/L for L and Z, respectively.

3.2.8 Statistical analysis

The statistical package IBM SPSS Statistics for Windows Version 22.0 was used for analysis. General linear models, with plasma L and Z as dependent variables, were the principal method of analysis. We included a core set of explanatory variables in all linear models, consisting of variables which had been identified in previous studies as being associated with plasma L and Z: age, sex, BMI, highest level of education (primary/none, secondary, and third level), smoking status (never, past or current smoker), and family history of AMD (yes, no and don't know). Controlling for these core variables, we also included the following explanatory variables, one at a time, in the general linear models: geographic location (Dublin city/county, another city or town, and rural), plasma cholesterol (TC, HDL, LDL, and triglyceride), alcohol consumption (number of drinks per week), physical exercise derived from the International Physical Activity Questionnaire-short form (inactive [low], minimally active [medium] and health enhancing physical exercise [high]), food supplement use (number of food supplements taken on a regular basis), and self-reported high blood pressure (yes/no). The 5% level of significance was used throughout, without adjustment for multiple testing.

3.3 Results

After excluding participants with AMD or self-reported eye disease (diabetic maculopathy, diabetic retinopathy, cataracts or glaucoma), data from 3,681 participants (99% of whom were white, mainly native Irish) were available for analysis (Figure 3.1). Demographic characteristics for these 3,681 participants studied are reported in Table 3.1. Of note, the 50-64 age group (70% of our sample), females (53% of our sample) and third level educated respondents (32% of our sample) are all over-represented, relative to the population from which the sample was drawn.

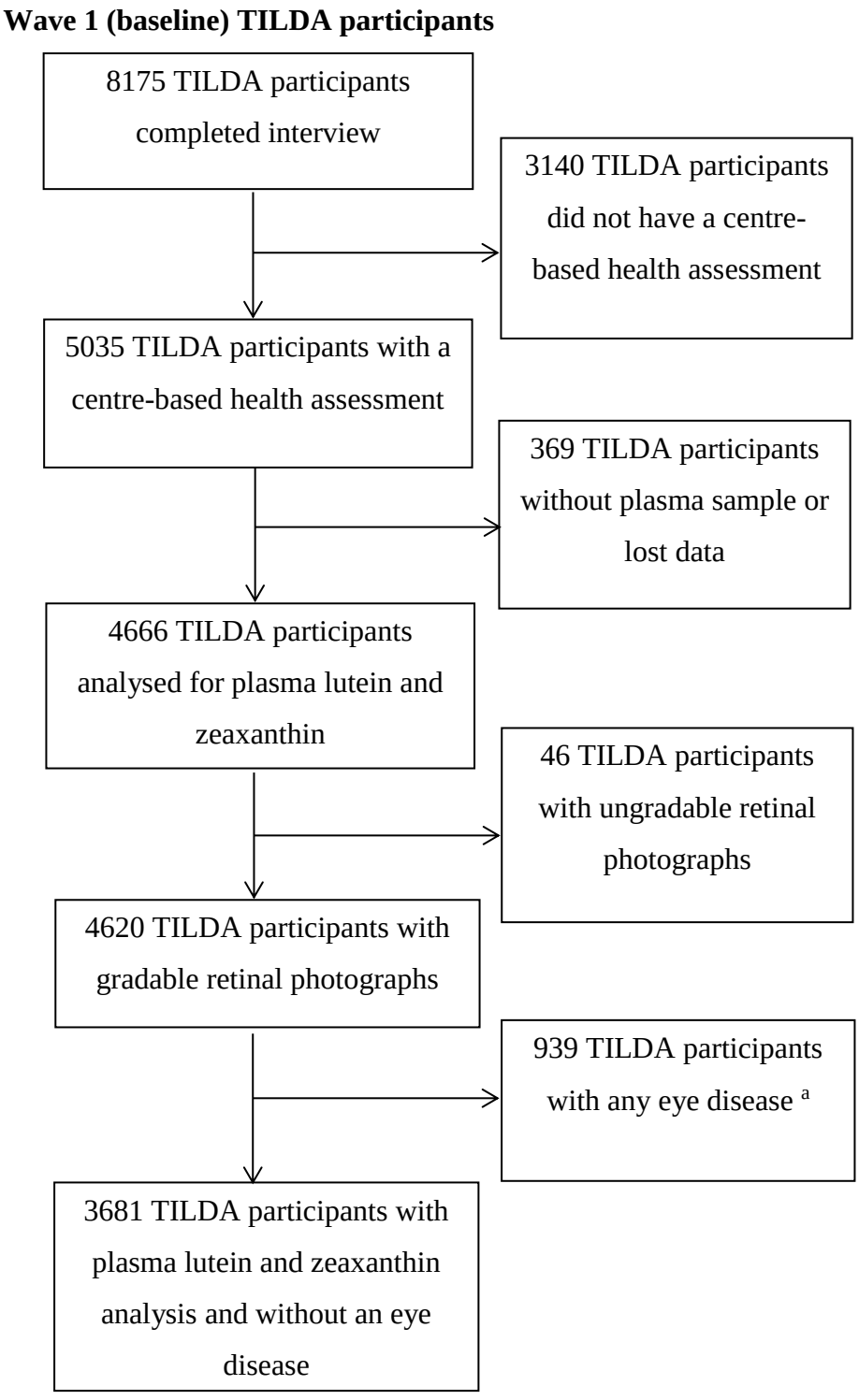

Figure 3.1: The Irish Longitudinal Study on Ageing (TILDA) participants included in this investigation; [a] any eye disease includes age-related macular degeneration (AMD) and self-reported: diabetic maculopathy, diabetic retinopathy, cataracts or glaucoma.

Table 3.1: Demographic, health and lifestyle characteristics of TILDA participants in this investigation.

Variable (n=3681)	**Mean ± SD or n (%)**
Age (years)	60.68 ± 7.70
50-64	2577 (70%)
65-74	898 (24.4%)
75+	206 (5.6%)
Sex (Female)	1955 (53.1%)
Education level	
Primary/none	744 (20.2%)
Secondary	1575 (42.8%)
Third level	1362 (37.0%)
BMI (Kg/m^2)	27.45 ± 4.90
Total Cholesterol, mmol/L	5.185 ± 1.050
HDL, mmol/L	1.556 ± 0.436
LDL, mmol/L	2.960 ± 0.940
Smoking status *	
Never/past smoker	3110 (84.5%)
Current smoker	560 (15.2%)
MP Optical Density (0.5°)	0.208 ± 0.159
Plasma L, µmol/L	0.2047 ± 0.115
Plasma Z, µmol/L	0.0567 ± 0.047
Exercise (per week) *	
Low	990 (26.9%)
Moderate	1314 (35.7%)
High	1377 (37.4%)
Family History of AMD *	184 (5.0%)

Data displayed are mean ± standard deviation (SD) for interval data and percentages for categorical data: Variables, variables analysed in the study; n, number of participants; Education level, highest level of education (primary/none, secondary, and third level); BMI, body mass index; Cholesterol, total cholesterol, high density lipoprotein (HDL), and low density lipoprotein (LDL); Smoking status, never-smokers (non-smoker), past smoker and current smoker; MP Optical Density, measured by customized heterochromatic flicker photometry at 0.5°; Plasma L and Z, lutein and zeaxanthin concentrations measured by high performance liquid chromatography, Exercise, % exercise per week (inactive [low], minimally active [medium] and health enhancing physical exercise [high]); Family history of AMD, % of participants self-reporting family history of age-related macular degeneration; *Self-reported.

Health and lifestyle variables are also presented in Table 3.1. Mean plasma L and Z concentrations were 0.2047 ± 0.115 μmol/L and 0.0567 ± 0.047 μmol/L as shown in Figure 3.2 and Figure 3.3, respectively.

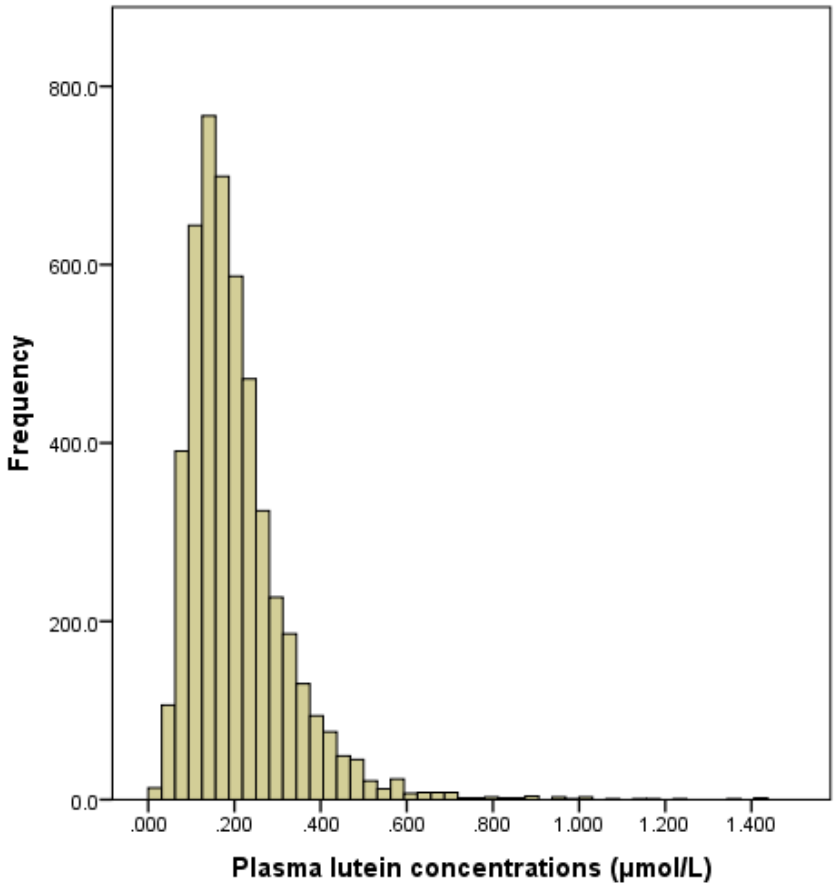

Figure 3.2: Histogram of plasma lutein concentrations in 3,681 TILDA participants in this investigation.

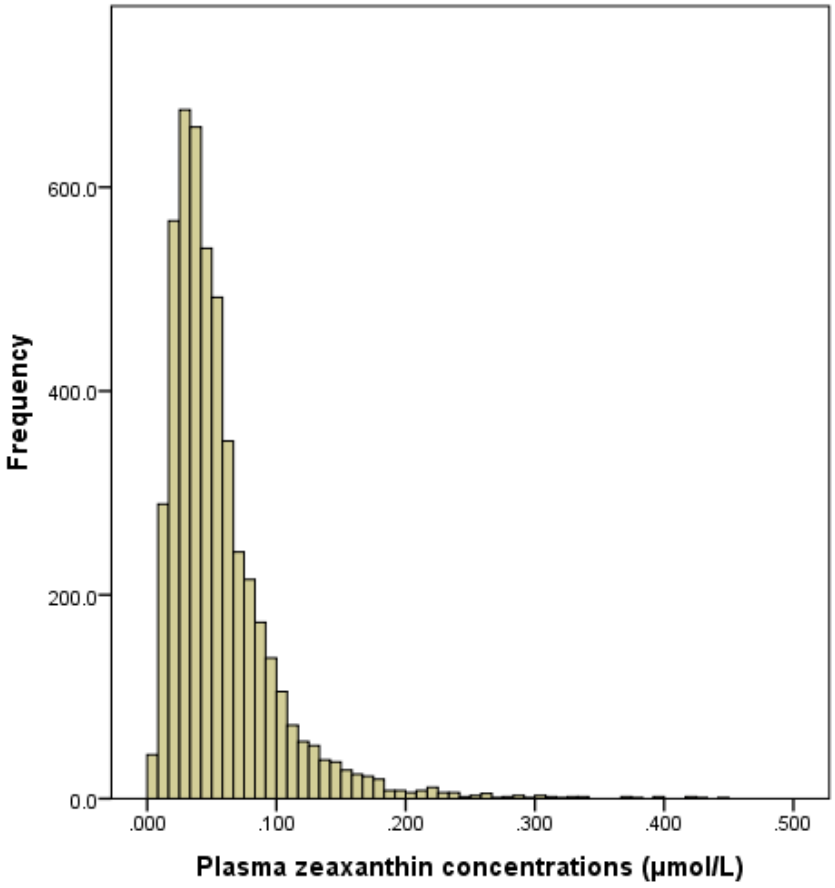

Figure 3.3: Histogram of plasma zeaxanthin concentrations in 3,681 TILDA participants in this investigation.

Plasma concentrations of L and Z were highly correlated with each other; Pearson correlation: $r = 0.647$, $p<0.001$ (Figure 3.4).

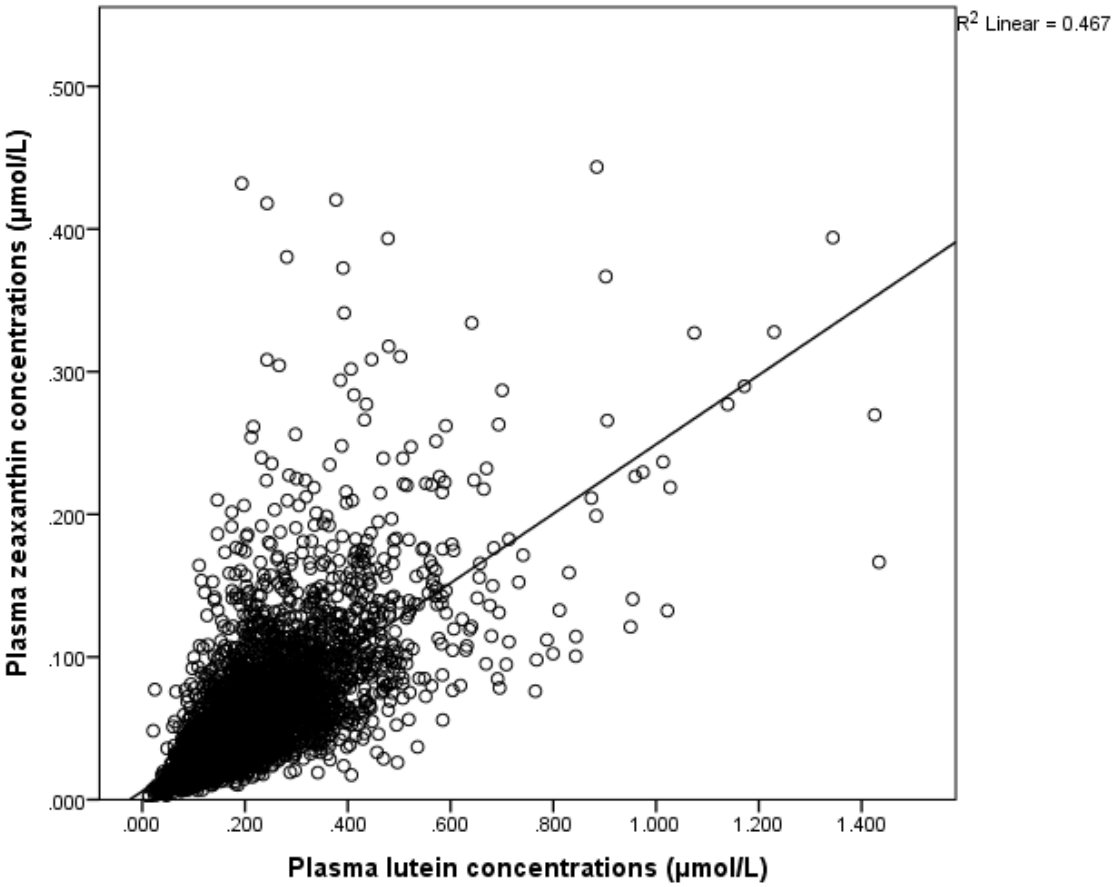

Figure 3.4: The relationship between plasma concentrations of lutein and zeaxanthin in 3,681 TILDA participants in this investigation.

There was also a positive and significant relationship between plasma concentrations of each of L and Z, and MP optical density (Figure 3.5 and Figure 3.6); Pearson correlation: r = 0.242, p<0.001 and r = 0.213 p<0.001, respectively.

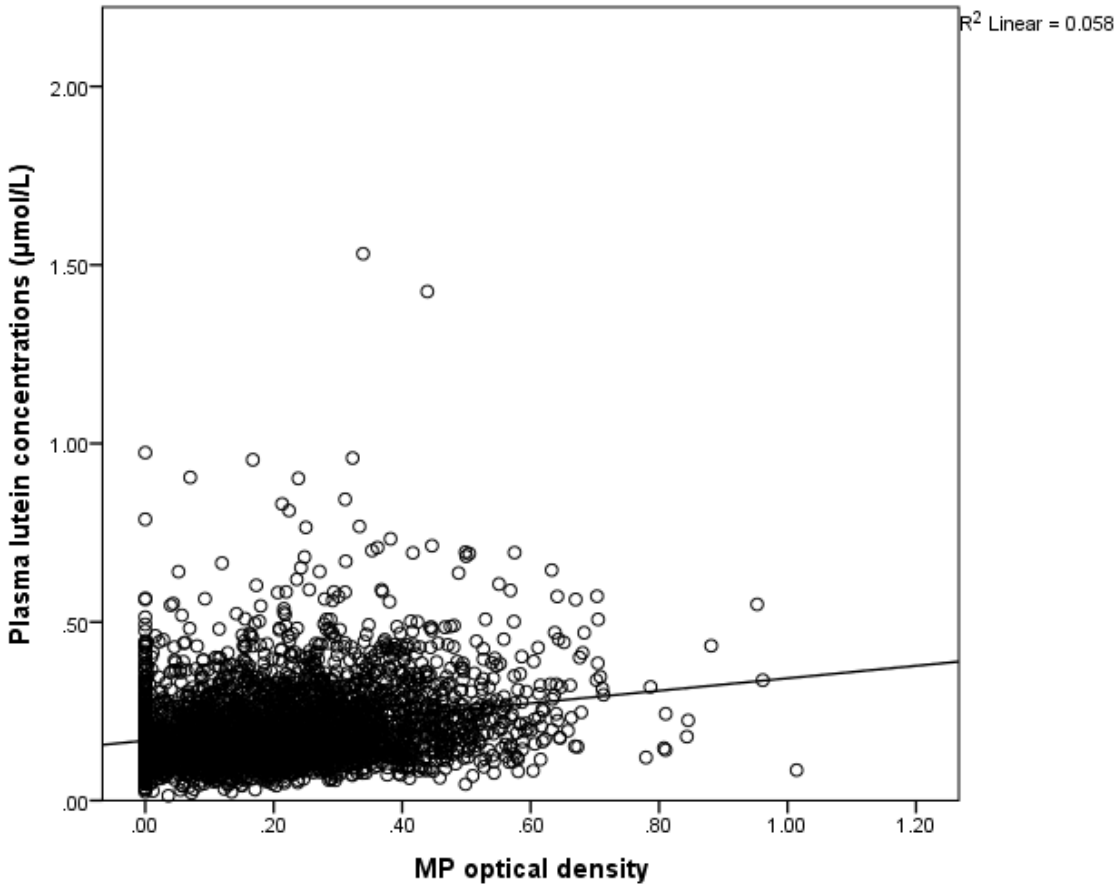

Figure 3.5: The relationship between plasma concentrations of lutein and macular pigment optical density (MPOD) of 3,681 TILDA participants in this investigation.

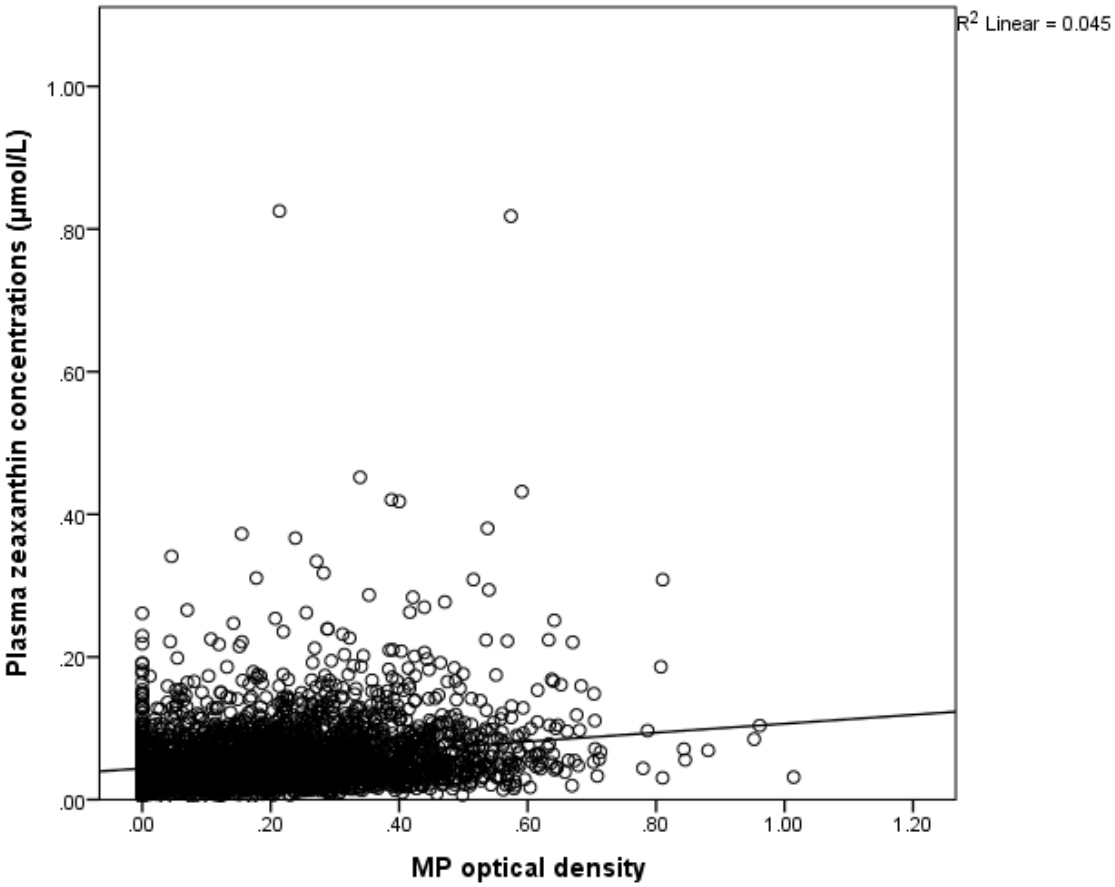

Figure 3.6: The relationship between plasma concentrations of zeaxanthin and macular pigment optical density (MPOD) of 3,681 TILDA participants in this investigation.

In the general linear model with putative core explanatory variables (age, sex, MP, education level, BMI, smoking status and family history of AMD), these variables were significantly related to plasma L after controlling for the other variables, with highly significant relationships ($p<0.001$) for sex, BMI, education level, family history of AMD, MP, and smoking status. Most of these core variables were also significantly related to plasma Z ($p<0.001$), the exceptions being age and family history of AMD. Table 3.2 and Table 3.3 summarise these findings. Of note, after the inclusion of food supplement use as a potential explanatory variable, these significant relationships persisted.

Table 3.2: Demographic and lifestyle variables as correlates and determinants of plasma lutein and zeaxanthin concentrations.

	Mean ± SD [a]					Mean ± SD [a]					Mean ± SD [a]			
	Lutein	**Sig.**	**Zeaxanthin**	**Sig.**		**Lutein**	**Sig.**	**Zeaxanthin**	**Sig.**		**Lutein**	**Sig.**	**Zeaxanthin**	**Sig.**
Sex					**Smoking Status**					**Education level**				
Male	0.1869 ± 0.098	<0.001	0.0515 ± 0.038	<0.001	*Never*	0.2153 ± 0.126	<0.001	0.0607 ± 0.052	<0.001	*Primary/none*	0.1837 ± 0.106	<0.001	0.0450 ± 0.034	<0.001
Female	0.2203 ± 0.127	-	0.0614 ± 0.053	-	*Past*	0.2059 ± 0.111	<0.001	0.0563 ± 0.045	<0.001	*Secondary*	0.1974 ± 0.113	<0.001	0.0553 ± 0.047	<0.001
					Current	0.1690 ± 0.086	-	0.0461 ± 0.031	-	*Third/higher*	0.2245 ± 0.121	-	0.0649 ± 0.051	-
Age					**Exercise**					**Family history of AMD**				
50-64	0.2051 ± 0.112	0.055	0.0594 ± 0.049	0.299	*Low*	0.1913 ± 0.105	0.002	0.0517 ± 0.045	0.004	*Yes*	0.252 ± 0.163	-	0.066 ± 0.004	-
65-74	0.1195 ± 0.110	0.018	0.0495 ± 0.037	0.197	*Medium*	0.2105 ± 0.120	0.197	0.0579 ± 0.048	0.183	*No*	0.203 ± 0.113	0.001	0.057 ± 0.0008	0.495
≥ 75	0.2215 ± 0.167	-	0.0554 ± 0.051	-	*High*	0.2092 ± 0.119	-	0.0594 ± 0.048	-	*Don't know*	0.194 ± 0.102	<0.001	0.052 ± 0.003	0.074

Data displayed are mean ± standard deviation (SD); [a] data expressed as µmol/L, plasma L and Z, concentrations measured by high performance liquid chromatography; Sig, significance difference between groups (dashes indicate reference group e.g. p values for education for comparison of primary and secondary educated subjects with third level educated subjects); smoking status, never-smokers (non-smoker), past smoker and current smoker; exercise, % exercise per week(inactive [low], minimally active [medium] and health enhancing physical exercise [high]); education level, highest level of education (primary/none, secondary, and third level); family history of AMD, % of participants self-reporting family history of age-related macular degeneration (AMD).

Table 3.3: Health variables as correlates and determinants of plasma lutein and zeaxanthin concentrations.

	Plasma lutein concentrations (Mean ± SD) [a]				**Plasma zeaxanthin concentrations (Mean ± SD) [a]**			
	1st tertile group	2nd tertile group	3rd tertile group	Sig.	1st tertile group	2nd tertile group	3rd tertile group	Sig.
BMI	0.2344 ± 0.139	0.2038 ± 0.105	0.1755 ± 0.091	<0.001	0.0648 ± 0.055	0.0574 ± 0.047	0.0480 ± 0.035	<0.001
MPOD	0.1770 ± 0.098	0.1970 ± 0.106	0.2379 ± 0.130	<0.001	0.0478 ± 0.035	0.0539 ± 0.044	0.0688 ± 0.056	<0.001
TC	0.1751 ± 0.095	0.2036 ± 0.116	0.2381 ± 0.127	<0.001	0.0453 ± 0.039	0.0554 ± 0.041	0.0706 ± 0.056	<0.001
HDL	0.1690 ± 0.091	0.1993 ± 0.101	0.2478 ± 0.137	<0.001	0.0469 ± 0.043	0.0549 ± 0.043	0.0691 ± 0.051	<0.001
LDL	0.1869 ± 0.112	0.2054 ± 0.113	0.2228 ± 0.118	<0.001	0.0494 ± 0.044	0.0559 ± 0.042	0.0653 ± 0.053	<0.001

Data displayed are mean ± standard deviation (SD); [a] data expressed as µmol/L, plasma L and Z, concentrations measured by high performance liquid chromatography; Sig, significance difference between groups; BMI, body mass index (kg/m^2); MPOD, macular pigment optical density 5˚; TC, total cholesterol; HDL, high density lipoprotein; LDL, low density lipoprotein.

As seen in Table 3.2, females and third level educated participants have significantly higher plasma L and Z concentrations, on average, compared to males and lower educated participants; current smokers have significantly lower average L and Z concentrations compared to past smokers and non-smokers. As seen in Table 3.3, plasma L and Z concentrations were significantly higher in the low and medium BMI tertile groups when compared to the high BMI tertile group, and were also significantly higher in the medium and high MP tertile groups when compared to the low MP tertile group.

The following potentially confounding variables were then added to model (which continued to include the core variables), one at a time, with the following statistically significant and positive results: total cholesterol ($p<0.001$, for both plasma L and Z models), HDL ($p<0.001$, for both plasma L and Z models), LDL ($p<0.001$, for both plasma L and Z models). Physical exercise was also significantly related to both plasma concentrations of L and Z, reflected in significantly lower plasma concentrations of these carotenoids amongst participants in the lowest exercise group when compared to the highest exercise group ($p<0.005$). These results are summarised in Table 3.2 and Table 3.3.

After controlling for the core variables, alcohol consumption was not significantly related to plasma concentrations of L or Z ($p>0.05$, for both), whereas plasma triglycerides were significantly and positively related to plasma concentrations of Z ($p<0.001$) but not to plasma concentrations of L ($p=0.057$), and self-reported hypertension was positively and significantly related to plasma concentrations of Z ($p=0.042$) but not to plasma concentrations of L ($p=0.058$). Use of food supplements

was positively related to plasma concentrations of L (p=0.008) but not to plasma concentrations of Z (p=0.059); of note, after controlling for food supplement use, the significant positive association between plasma L concentrations and age remained. Finally, plasma concentrations of L were significantly lower amongst urban dwellers (another city/town, other than Dublin) versus dwellers of rural areas (p=0.001).

3.4 Discussion

To our knowledge, this is the largest study of its kind to report on the relationships between plasma concentrations of L and Z and non-dietary correlates and determinants of these carotenoids in an older Irish population. The main finding from our study is that plasma concentrations of L and Z are associated with the following variables: tobacco use, sex, BMI, education, physical exercise, cholesterol status, age, family history of AMD and MP levels. Importantly, the modifiable variables reported here are associated with lifestyle and behavioural habits, and we discuss these findings, and their implications, below.

Firstly, while the study design of TILDA is consistent with other population-based longitudinal studies,[45, 46] and while there are many similarities in terms of the main study variables analysed between those studies and ours, TILDA is unique, in that it contains data on plasma concentrations of L and Z *and* MP collected from a large, random, racially homogenous sample of older Irish adults. It is also a longitudinal study, but we report here baseline (cross-sectional) data from TILDA, comparing our findings to the findings of other cross-sectional studies, which also report on plasma

concentrations of L and Z in relation to other potentially explanatory variables in older adult populations. These studies include: NHANES III,[33] CAREDS,[32] the European Prospective Investigation into Cancer and Nutrition (EPIC),[31] the European Eye study (EUREYE),[30] and some other observational studies.[34-37] Of note, these studies were conducted between 1988 and 2004, and TILDA therefore represents an up-to-date (data for this report captured between 2009 and 2011) report on plasma concentrations of L and Z and their relationship with non-dietary variables. This is an important point, because some of the factors known to influence circulating concentrations of plasma L and Z have changed in recent years. For example, trends in tobacco use, lifestyle and dietary habits, and use of dietary supplements, have changed greatly since these earlier reports,[47, 48] and all these factors need to be taken into consideration when interpreting our findings.

The mean L and Z plasma concentrations reported in our study was comparable with the EUREYE study,[30] but was lower when compared to the EPIC study.[31] Possible reasons for the disparity between studies may be explained by differences in the populations studied (of note, the EPIC study was sampled across 16 regions of Europe), and because variables such as age, sex, diet and use of food supplements differ between samples studied.[30, 31]

Consistent with previous reports, we found that tobacco use is associated with significantly lower circulating plasma concentrations of L and Z.[33, 34] Indeed, current cigarette smokers exhibited 24% and 27% lower plasma concentrations of L and Z, respectively, when compared with non-cigarette smokers. This finding is unsurprising, because cigarette smokers have been shown to have diets lacking in fruits and

vegetables (the source of L and Z),[49, 50] but also because it has been shown that cigarette smokers have an increased overall oxidant load, thus reducing circulating plasma carotenoid concentrations in smokers.[51]

We also found a statistically significant inverse relationship between BMI (a measure of obesity) and plasma concentrations of L and Z, which is also consistent with previous reports.[30, 31, 34, 37, 52, 53] Of note, high BMI is known to be associated with increased oxidative stress.[54] Also, it has been shown that participants with high BMI (>25 kg/m^2) have poor diets lacking in fruits and vegetables,[55-58] which would also help explain our findings. Moreover, we know that body fat (i.e. adipose tissue) is a major storage site for carotenoids,[59] and it has been suggested that adipose tissue may compete with other tissue receptors (e.g. the retina) for uptake of the carotenoids from serum.[29, 60] Of note, our findings are consistent with the CAREDS study, which reported that women with high BMIs and high body fat exhibited lower circulating plasma L and Z concentrations when compared to participants with normal BMI and normal body fat.[32] This finding is also consistent with Kimmons *et al.* (NHANES III), who have shown that participants with high BMIs (overweight and obese) exhibit low serum carotenoid concentrations, when compared to participants with normal BMIs.[61]

Another finding from our study is that plasma L and Z concentrations were positively related to both HDL and LDL. This finding is not surprising, because carotenoids are transported in plasma by these lipoproteins, in a way that relates to the degree of hydrophobicity of those particles.[62] However, Clevidence *et al.* reported that HDL is the primary carrier of L and Z,[63] but our data shows that both HDL and LDL are comparable correlates (and possibly determinants) of plasma concentrations of L and Z,

suggesting that L and Z are transported on both lipoproteins. Of note, some previous studies have reported that serum concentrations of L and Z are positively and significantly related to HDL (but not to LDL),[64-66] while others have reported serum concentrations of L and Z to be positively related to HDL and to LDL,[37, 67, 68] the latter studies being consistent with our findings.

Consistent with the findings reported previously by our group (see Nolan *et al.*[36] and Loane *et al*.[65]), we found in the current TILDA sample that participants reporting a family history of AMD (n=185) had significantly higher plasma L concentrations when compared to participants with no known family history of AMD (n=3496). Of note, we found no relationship between plasma concentrations of Z and a reported family history of AMD, which is also consistent with the aforementioned studies.[32, 36, 65] Importantly, and similar to Nolan *et al*. and Loane *et al*., our study compares plasma concentrations of L and Z (separately) for participants with and without a confirmed family history of AMD. In contrast, the CAREDS study reported that a family history of AMD was not significantly related to serum concentrations of L and Z (combined) in elderly women. We believe reporting serum concentrations of L and Z (combined) could confound any potential association between serum concentrations of either of these carotenoids (in isolation) and potential correlates and determinants (e.g. age and positive family history of AMD as discussed in Chapter four). Our finding that participants with a reported family history of AMD exhibited significantly higher concentrations of L is important, and may reflect carotenoid supplement use in those with a family history of this condition.[69] Of note, the ROI is a small country with a population of only 4.6 million[70] with the prevalence of AMD in the population aged 50 years and older estimated at

7.2%.[9] Awareness of AMD in the ROI, and the potential benefits of supplementation with the macular carotenoids in patients at risk of AMD, have been discussed at length in newspapers, on radio and on Irish TV (e.g. RTE Nationwide), which has enhanced penetration of this small market by distributors of dietary supplements for eye health.

We report a statistically significant positive relationship between age and plasma L (i.e. older participants having higher plasma L concentrations); however, there was no significant association between age and plasma concentrations of Z. These findings may also reflect food supplement use, which is typically more common in the older population, and given that food supplements typically contain high amounts of L (and little or no Z) in their formulations;[71] however, when we controlled for food supplement use in this study, older participants still had significantly higher plasma L concentrations. Of note, there are a number of other studies that have reported on the relationship between age and serum concentrations of L and Z.[30, 33-35, 37, 72, 73] Indeed, our findings are consistent with those of Olmedilla-Alonoso *et al.*, who reported higher serum L concentrations in older adults compared to younger adults, and no association between plasma Z and increasing age.[74] Interestingly, O'Connell *et al.* found that increasing age was associated with reduced dietary intake of Z, but not with reduced dietary intake of L.[75]

Also, and consistent with other studies, we found that plasma concentrations of L and Z were higher in female participants when compared to male participants,[31, 34, 76] and higher in participants reporting more physical activities,[32] which may be explained, at least in part, by better dietary habits in female participants[35, 77] and those performing physical exercise.[34, 78] Other correlates of plasma L and Z concentrations in our study

include MP and education levels, each of which was significantly and positively related to plasma concentrations of these carotenoids, findings that are consistent with previous reports.[32, 74, 79, 80] For example, it has been previously shown that plasma L and Z concentrations are important determinants of MP, which is unsurprising given that retinal capture of circulating carotenoids is required to accumulate these nutrients at the macula.

Our finding that plasma concentrations of L and Z are related to education levels are consistent with those of Rock *et al.* and the EUREYE study, which reported that higher serum concentrations of L and Z were associated with third level education.[30, 34] Indeed, our findings that education level is a determinant of plasma concentrations of L and Z is also consistent with our previous report from this sample (see Nolan *et al*.[42]), which found that the level of education was positively and significantly related to MP. These findings are important, although unsurprising and intuitive, given that a lack of education is associated with many negative correlates (and possibly determinants) of plasma concentrations of L and Z including obesity, low physical exercise, tobacco use, and poor diet.[81, 82]

The main strengths of this study can be summarised as follows: it is a large population size (n=3963) randomly selected, racially homogeneous sample (99% white and of Irish birth). Furthermore, standardised methods (including blood processing and storage) and use of a single laboratory to carry out the carotenoid analysis minimised variability. The main limitations of the TILDA study were an underrepresentation of the age group of 75 years and older, overrepresentation of female participants, and underrepresentation of participants who attended primary level education compared

with the overall population of ROI.[83] Also, dietary data and additional information on food supplement use (ingredients for multivitamins, dose and dose regimen) would have allowed for more detailed analysis with respect to correlates (and putative determinants) of plasma L and Z concentrations in the Irish population.

3.5 Conclusion

We reported on the demographic, lifestyle and health status of 3,681 Irish adults, in order to investigate correlates and identify potential determinants of plasma concentrations of L and Z. The findings of this large study indicated that plasma concentrations of L and Z are lower in association with indicators of a poor lifestyle (high BMI, tobacco use, and less physical exercise) and lower education, indicating that modifying lifestyle in a positive way is likely to be reflected in higher concentrations of plasma carotenoids with consequential health benefits. Of the determinants and correlates that were investigated in this chapter, up to 18% and 13.3% of variance in plasma L and Z, respectively, can be explained by independent variables (sex, education, age, family history of AMD, MP, BMI, smoking status and total cholesterol). This was reasonably comparable to other studies (24% and 13%, respectively).[33, 34] This work was published in the *Journal of Nutrition, Health and Aging* in 2016, under the title, "Non-dietary correlates and determinants of plasma lutein and zeaxanthin concentrations in the Irish Population" (Appendix D).

Chapter 4: Self-reported and actual prevalence of age-related macular degeneration in a population based sample: implications for research and policy in public health ophthalmology

4.1 Introduction

The macula, a specialised area of the retina, mediates central and colour vision.[84] AMD is a disease of the macula that, in its advanced stage, results in the loss of central vision if untreated or if untreatable.[85] Early (non-advanced) AMD is characterised by drusen and/or pigmentary abnormalities, whereas the late (advanced) form of AMD is visually consequential and can be classified as atrophic ([GA or dry] or [CNV or wet]).[86] AMD is the leading cause of irreversible blindness in the older population, especially in developed countries.[87] We have recently shown that the overall prevalence of any form of AMD (i.e. early or advanced) in adults aged 50 years or older in ROI is 7.2% (census-weighted).[9] The incidence and prevalence of AMD will continue to rise because of increasing longevity and because of the growing world population.[88] The global projection of people with AMD is estimated at 196 million by 2020, further increasing to 288 million by 2040.[89]

The loss of central vision in patients afflicted with AMD has a dramatic and adverse impact on their quality of life.[90] For example, the impact of vision loss associated with AMD may result in an inability to drive, to read, to recognise faces, or to watch television, with a consequential loss of social independence and increasing need for family support,[91] which is a major concern in the context of an advancing population. The financial burden of vision loss and/or impairment may be classed as

direct or indirect.[92] The indirect costs include the loss of income (by the patient), the cost of care-givers, nursing homes and other costs (e.g. transport, etc.).[92] Direct costs include hospital care, outpatient and office visits, optometry costs, drugs and other direct expenses.[92] Currently, there is no effective treatment for atrophic AMD, whereas neovascular AMD is treated by intravitreal injections of anti-VEGF therapy, which has been shown to dramatically reduce the risk of vision loss,[93] but at an average annual cost per eye per year of $24,000.[94]

Established risk factors for AMD include increasing age, family history of disease (genetic background), and tobacco use; whereas exposure to short-wavelength (blue) light, obesity, cardiovascular disease and diet (antioxidant status) are described as putative risk factors for this condition.[95] Although the aetiopathogenesis of AMD remains elusive, we now know that oxidative stress is a key factor in the development of this disease.[96, 97] Indeed, and over the last few decades, there has been a growing body of research investigating the protective role of carotenoid antioxidants for AMD,[98] which culminated in the publication of AREDS 2 in 2013.[99, 100] Specifically, it has been demonstrated that supplementation with at least two of the three macular carotenoids (L and Z) in association with co-antioxidants (vitamin C, vitamin E, zinc, and copper) reduces the risk of progression from intermediate AMD to advanced AMD.[99, 100] Moreover, a recent double-blind, randomised clinical trial reported that, in patients with early AMD, supplementation with all three of MP's constituent carotenoids in MZ:L:Z (mg) ratio of 10:10:2 enhances visual performance and is non-inferior (in terms of MP augmentation) to the AREDS 2 formulation.[101] Interestingly, carotenoids are entirely of dietary origin and, therefore, their concentrations in plasma and consequential

bioavailability for uptake by the retina is dependent on consumption of foods containing these nutrients (such as leafy greens and coloured fruits and vegetables) or supplements.

The association between plasma concentrations of L and Z with diet, age, ethnicity, and AMD status has been previously reported.[38, 53, 102, 103] However, the current investigation, conducted as part of TILDA study, see below, is the first population-based study to report on the relationship between plasma concentrations of L and Z and grading-confirmed AMD while investigating the impact of self-reporting of AMD, supplement use and plasma concentrations of the relevant carotenoids in the ROI.

4.2 Methods

4.2.1 Study design

TILDA is a nationally representative, longitudinal study of the health, economic and social status of 8,175 adults aged 50 years and over in the ROI. At the time of the study reported herein, the total number of adults aged 50 years and over in the ROI was *circa* 1.2 million.[83] The design and methodology of TILDA are described in detail elsewhere.[39] In brief, a nationally representative sample of community-dwelling adults was drawn from the Irish Geodirectory, a comprehensive record of all residential addresses in the ROI. Addresses were selected by means of RANSAM using a three stage process where all household residents aged 50 years or older were eligible to participate.[39] As the name of the study suggests, TILDA is a longitudinal study, which began in 2009 (with interviews every two years and health assessments every four

years), but the focus of this report relates only to baseline (wave 1) data (collected between 2009 and 2011).

4.2.2 Participants

Wave 1 consisted of three separate components: 1) a face-to-face interview using a CAPI carried out in the participant's own home; 2) a SCQ; 3) a health assessment carried out in either a dedicated health centre (based in Dublin and Cork) or, alternatively, a modified assessment was carried out in the participant's own home if he/she was unable/unwilling to travel to the health centre. Retinal photographs were not obtained from participants who opted not to attend a health centre assessment (n=3140). Wave 1 health assessment had an overall response rate of 62% (i.e. 62% of 8,175 = 5035). Figure 4.7 illustrates TILDA participants included in this investigation.

This study was approved by the Faculty of Health Sciences Research Ethics Committee of Trinity College Dublin and the local Ethics Committee at the Waterford Institute of Technology. All participants provided signed informed consent prior to enrolment in the study. Separate written and verbal consent was required to obtain and store blood samples from participants. The study was conducted in accordance with the tenets of the Declaration of Helsinki regarding research into human volunteers.

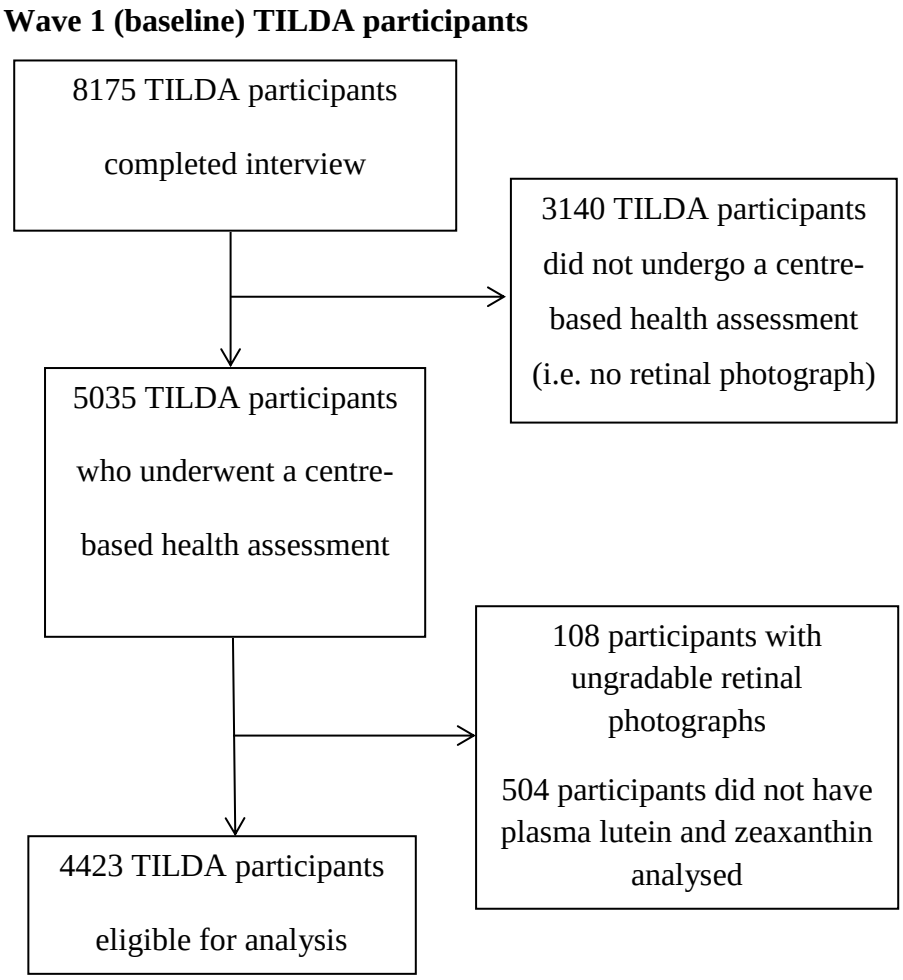

Figure 4.7: The Irish Longitudinal Study on Ageing (TILDA) participants included in this investigation. Photographs were judged as ungradable based on a priori criteria of photographic quality. The phenomenon of participants without plasma concentrations of lutein and zeaxanthin was attributable to one of the following: participant did not consent to give a blood sample, a failure to perform a venepuncture at the time of the health assessment, insufficient sample for carotenoid analysis or sample lost during carotenoid extraction/analysis.

4.2.3 Interview

Participants completed a CAPI, which was carried out by a trained social interviewer.[39] Participants were asked whether, to their knowledge, a doctor's diagnosis of AMD had been made in their individual case and, whether, to their knowledge, there was a family history of AMD. A list of medications, including food supplements consumed on a daily basis, was recorded for each participant. The exact wording of the question was as follows: "MD001: Now I would like to record all medications that you take on a regular basis, like every day or every week. This will include prescription and non-prescription medications, over-the-counter medicines, vitamins, and herbal and alternative medicines." For the purpose of the current analysis, information on supplements that contained at least one of the three constituent carotenoids of MP (i.e. either L and/or Z and/or MZ) was also recorded, and this was then coded as yes or no (and henceforth referred to as supplement use).

4.2.4 Retinal photographs

All retinal photography was performed by TILDA nurses, who were trained and certified by experts from the Ocular Epidemiology Reading Centre at the University of Wisconsin, Madison, USA. One 45° monoscopic colour photograph, centred on the macula (EDTRS standard field 2) was obtained for each eye using the NIDEK AFCE-210 non-mydriatic auto-fundus camera through a non-dilated pupil. Pupil dilation was not feasible for this large study, given that participants had to undergo many other tests, some of which would have been adversely influenced by pupil dilation (e.g. gait assessment).

4.2.5 AMD grading

Retinal photographs were graded using a modified version of the International Classification and Grading System for AMD.[9] Early (non-advanced) AMD was defined as the presence of more than 10 hard drusen (< 63µm) and/or the presence of soft drusen (>125 µm). Late (advanced) AMD was defined as the presence of atrophic AMD and/or neovascular AMD.

4.2.6 Plasma L and Z assessment

The TILDA protocol for non-fasting venous blood sample collection, processing and storage has been described previously.[104] In brief, 1 ml of plasma wrapped in tinfoil was stored at -80°C and dedicated to carotenoid assessment. L and total Z (Z and MZ) was analysed using a reversed phase HPLC method. Details of the extraction procedures and HPLC analysis that we used has been previously described.[105] Plasma concentrations of L and Z were measured in non-fasting plasma samples and therefore could be influenced by recent dietary intake of these nutrients.

4.2.7 Statistical analysis

The statistical package IBM SPSS Statistics for Windows Version 22.0 was used for analysis. In an earlier report of this TILDA cohort,[9] we found that AMD prevalence was linked to age and family history of AMD. In the current analysis, we investigated the respective relationships between self-reported AMD (justified and unjustified), use of supplements containing at least one of the three macular carotenoids and mean plasma concentrations of L and Z. The statistical methods employed included contingency table

analysis and post hoc analysis of variance. Accordingly, and along with plasma concentrations of L and Z, we incorporated these variables into our logistic regression models for this current study. However, we did not incorporate other variables, such as sex, education, BMI etc., as these were not found to be significantly related to AMD in the earlier report of this cohort).[9] The 5% level of significance was used throughout, without adjustment for multiple testing.

4.3 Results

Demographic, health and lifestyle characteristics for the 4,423 TILDA participants reported herein are presented in Table 4.4.

Table 4.4: Demographics, health and lifestyle characteristics of participants in this investigation.

Variables (n=4423)	**Mean ± SD or n (%)**
Age (years)	61.36 ± 8.19
50-64	2888 (65.3%)
65-74	1174 (26.5%)
75+	361 (8.2%)
Sex (Female)	2378 (53.8%)
Education level	
Primary/none	944 (21.4%)
Secondary	1861 (42.1%)
Third level	1616 (36.5%)
BMI (kg/m^2)	27.13 ± 8.13
Smoking status*	
Never	2036 (46.0%)
Past	1728 (39.1%)
Current	659 (14.9%)
Plasma L, µmol/L	0.2072 ± 0.128
Plasma Z, µmol/L	0.0562 ± 0.047
Family history of AMD*	
Don't know	423 (9.6%)
No	3769 (85.2%)
Yes	231 (5.2%)
Self-reported AMD*	65 (1.5%)
Grading-confirmed AMD	288 (6.5%)
Early AMD	273 (6.2%)
Late AMD	15 (0.34%)
Supplement use*	102 (2.3%)

Data displayed are mean ± standard deviation (SD) for interval data and percentages for categorical data: Variables, variables analysed in the study; n, number of participants; Education level, highest level of education (primary/none, secondary, and third level); BMI, body mass index; Smoking status, never (non-smoker), past smoker and current smoker; Plasma lutein (L) and total zeaxanthin (Z), concentrations measured by high performance liquid chromatography; Family history of AMD, % of participants self-reporting family history of age-related macular degeneration (AMD); Self-reported AMD, % of participants that self-reported a doctor diagnosis of AMD; Grading-confirmed AMD, % of participants with grading-confirmed AMD, retinal photographs were graded by a certified grader using a modified version of the International Classification and Grading System for AMD, thereby establishing such a diagnosis; Supplement use, use of supplements containing either L and/or Z and/or MZ;* Self-reported.

Of note, among those who did not attend the health centre assessment, 79 participants (2.52%) self-reported their eyesight as poor compared to 52 participants (1.03%) who attended the health centre and self-reported their eyesight as poor. Among those who did not attend the health centre assessment, 57 participants (1.8%) self-reported a doctor diagnosis of AMD compared to 84 participants (1.67%) who attended the health centre and self-reported a doctor diagnosis of AMD (p=0.617).

The participants' mean age was 61 ± 8 years; and 231 participants (5.2%) reported a family history of AMD. 288 participants (6.5%) exhibited signs of early or late AMD (early AMD: n=273; 6.2%; late AMD: n=15; 0.34%;). Plasma concentrations of L were significantly higher in participants with late AMD (p<0.001) (Table 4.5). Plasma concentrations of Z were not significantly different in the three groups (p=0.317) (Table 4.5).

Table 4.5: Mean plasma concentrations of lutein and zeaxanthin for disease-free, early and late AMD groups.

AMD status	Mean plasma lutein ± SD	Mean plasma zeaxanthin ± SD
Disease-free (n=4135)	0.2058 ± 0.125	0.0562 ± 0.048
Early AMD (n=273)	0.2313 ± 0.176	0.0591 ± 0.052
Late AMD (n=15)	0.3461 ± 0.189	0.0712 ± 0.038

Data displayed are mean ± standard deviation (SD); AMD status, retinal photographs were graded by a certified grader using a modified version of the International Classification and Grading System for age-related macular degeneration (AMD); plasma lutein and zeaxanthin expressed as µmol/L, concentrations measured by high performance liquid chromatography.

Because of the small numbers of cases of late AMD, cases of early (non-advanced) and late AMD were combined in subsequent analyses (and henceforth referred to as AMD). As previously reported in this sample,[9] increasing age and a positive family history of AMD were strongly associated with prevalence of AMD.

Using logistic regression, the association between grading-confirmed AMD and plasma concentrations of L and Z, in each case controlling for age and family history of AMD, were investigated. The relationship between AMD and plasma L was positive and highly significant ($p<0.001$), demonstrating that AMD is associated with *higher* plasma concentrations of L in this cohort. There was no significant relationship between plasma concentrations of Z and AMD ($p>0.05$). For completeness, the logistic regression model was also fitted with putative core explanatory variables (BMI, sex, education, smoking status, cholesterols [TC, HDL and LDL] and physical exercise) along with age and family history of AMD, plasma L (but not Z) was still significantly associated with AMD.

This unexpected association between AMD and plasma concentrations of L prompted us to explore whether self-reported family history of AMD and/or self-report of AMD (whether justified or not) were associated with use of supplements containing at least one of the three macular carotenoids, which in turn might have contributed to our observations. In our study cohort, 231 participants (5.2%) reported a family history of AMD and 102 participants (2.3%) reported use of supplements containing at least one of the three macular carotenoids (supplement use). A small number of participants (n= 65; 1.5%;) believed that they were afflicted with AMD on the basis of a doctor's diagnosis, whereas 4,358 participants (98.5%) reported that they were not afflicted with

this condition. Subsequent AMD grading, however, revealed that not all of these self-reports were accurate, and that we had, in fact, four distinct subject groups in our study: Group 1 (n=24): grading-confirmed AMD in association with justified self-report of AMD (i.e. grading positive/self-report positive); Group 2 (n=264): grading-confirmed AMD in participants who were unaware that they suffer from AMD (i.e. grading positive/self-report negative); Group 3 (n=41): grading-confirmed absence of AMD in participants who (incorrectly) self-reported AMD (i.e. grading negative/self-report positive); Group 4 (n=4094): grading-confirmed absence of AMD in participants who justifiably reported that they were not afflicted with AMD (i.e. grading negative/self-report negative) (Table 4.6).

Table 4.6: Groups of participants in relation to grading-confirmed AMD and self-report of AMD.

Group 1 (n=24)	Grading-confirmed AMD and self-reported (aware) having AMD
Group 2 (n=264)	Grading-confirmed AMD and self-reported not having (unaware) AMD
Group 3 (n=41)	Grading-confirmed absence of AMD however self-reported having AMD
Group 4 (n=4094)	Grading-confirmed absence of AMD consistent with participant self-report

Grading-confirmed age-related macular degeneration (AMD), retinal photographs were graded by a certified grader using a modified version of the International Classification and Grading System for AMD; Self-report of AMD, participants were asked whether a doctor had diagnosed them with AMD; n= number of participants.

Analysis of variance was used to compare mean plasma concentrations of L and Z in these four groups ($p<0.001$), for both (see Table 4.7 and Figure 4.8). Post hoc analysis of variance (Tukey HSD) was also performed for comparison of groups in pairs

(Appendix E). Group 1 (grading positive/self-report positive), Group 3 (grading negative/self-report positive) and Groups 2 and 4 combine (grading positive/self-report negative and grading negative/self-report negative) were identified as being significant different from each other with respect to plasma concentrations of L. Only Group 1 (grading positive/self-report positive) was identified in the Tukey analysis as being significant different from the other groups with respect to plasma concentrations of Z.

Table 4.7: Comparison of plasma concentrations of lutein and zeaxanthin in groups.

						95% Confidence Interval for Mean	
	Group	**n**	**Mean**	**±SD**	**±SE**	**Lower Bound**	**Upper Bound**
Plasma L (µmol/L)	1	24	0.4691	0.3720	0.0759	0.3120	0.6262
	2	264	0.2162	0.1317	0.0081	0.2003	0.2322
	3	41	0.3176	0.2354	0.0367	0.2433	0.3919
	4	4094	0.2040	0.1281	0.0019	0.2035	0.2077
Plasma Z (µmol/L)	1	24	0.1101	0.1045	0.0213	0.0659	0.1542
	2	264	0.0551	0.0409	0.0025	0.0502	0.0601
	3	41	0.0735	0.0691	0.0108	0.0517	0.0954
	4	4094	0.0557	0.0460	0.00072	0.0543	0.0572

Data displayed are mean ± standard deviation (SD) and standard error (SE); plasma lutein and zeaxanthin expressed as µmol/L, concentrations measured by high performance liquid chromatography; n= number of participants; grading-confirmed age-related macular degeneration (AMD), retinal photographs were graded by a certified grader using a modified version of the International Classification and Grading System for AMD; Group 1 (n=24): grading-confirmed AMD in association with self-reported AMD; Group 2 (n=264): grading-confirmed AMD in the absence of self-reported AMD; Group 3 (n=41): grading-confirmed absence of AMD in association with self-reported AMD; Group 4 (n=4094): grading-confirmed absence of AMD in association with self-reported absence of AMD.

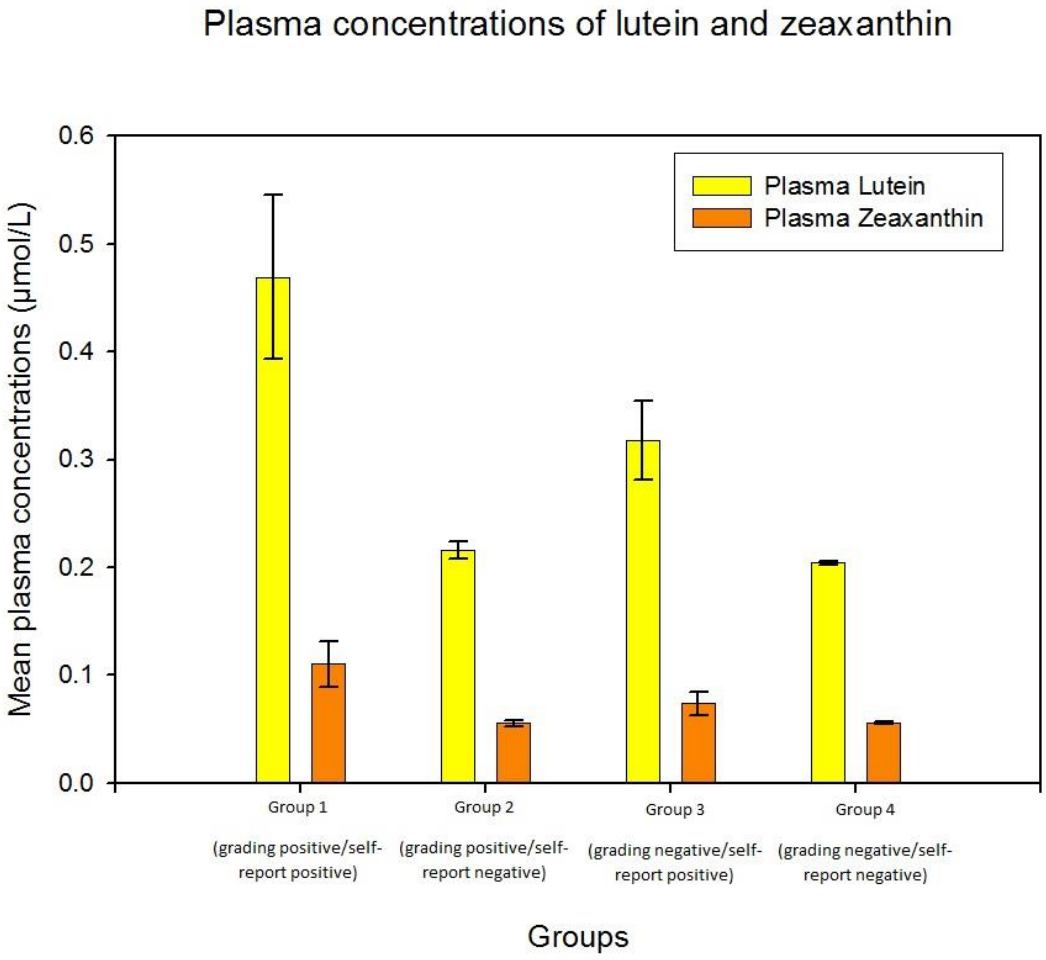

Figure 4.8**:** Mean plasma concentrations of lutein and total zeaxanthin of groups in this investigation. Plasma lutein and zeaxanthin concentrations measured by high performance liquid chromatography. Group 1 (n=24): grading-confirmed age-related macular degeneration (AMD) in association with self-reported AMD; Group 2 (n=264): grading-confirmed AMD in the absence of self-reported AMD; Group 3 (n=41): grading-confirmed absence of AMD in association with self-reported AMD; Group 4 (n=4094): grading-confirmed absence of AMD in association with self-reported absence of AMD; grading-confirmed AMD, retinal photographs were graded by a certified grader using a modified version of the International Classification and Grading System for AMD; self-reported AMD, participants were asked whether a doctor had diagnosed them with AMD.

The role of other variables that might have influenced the observed relationships between a grading-confirmed diagnosis of AMD and plasma concentrations of L were also investigated, including use of supplements containing at least one of the three macular carotenoids (supplement use) and factors that might have prompted the use of such supplements (i.e. family history of AMD and/or self-report of AMD).

Nearly half of the participants in Group 1 (grading positive/self-report positive) reported no family history of AMD. The proportion in Group 3 (grading negative/self-report positive) reporting a family history of AMD was significantly higher than in Groups 2 and 4 (grading positive/self-report negative and grading negative/self-report negative, respectively) ($p<0.001$, Pearson Chi-Square) (Table 4.8).

Table 4.8: Self-reported family history of AMD by group.

	Family history of AMD		
Groups	**Don't know (%)**	**No (%)**	**Yes (%)**
Group 1 (grading positive/self-report positive)	2 (8.3%)	11 (45.8%)	11 (45.8%)
Group 2 (grading positive/self-report negative)	24 (9.1%)	226 (85.6%)	14 (5.3%)
Group 3 (grading negative/self-report positive)	4 (9.8%)	22 (53.7%)	15 (36.6%)
Group 4 (grading negative/self-report negative)	393 (9.6%)	3510 (85.7%)	191 (4.7%)

Group 1 (n=24): grading-confirmed age-related macular degeneration (AMD) in association with self-reported AMD; Group 2 (n=264): grading-confirmed AMD in the absence of self-reported AMD; Group 3 (n=41): grading-confirmed absence of AMD in association with self-reported AMD; Group 4 (n=4094): grading-confirmed absence of AMD in association with self-reported absence of AMD; grading-confirmed AMD, retinal photographs were graded by a certified grader using a modified version of the International Classification and Grading System for AMD; self-reported AMD, participants were asked whether a doctor had diagnosed them with AMD; family history of AMD, participants self-reporting family history of AMD.

Supplement use was found to be significantly higher in participants who self-reported AMD (whether justified or unjustified); 41.7% and 17.1% of participants in Groups 1 and 3, respectively, were using a supplement containing at least one of the three macular carotenoids, and this compares with only 2.7% and 1.9% of Groups 2 and 4, respectively ($p<0.001$, Pearson Chi-Square) (Table 4.9).

Table 4.9: Lutein and/or zeaxanthin and/or meso-zeaxanthin supplement use by group.

	L and/or Z and/or MZ supplement use	
Groups	**No (%)**	**Yes (%)**
Group 1 (grading positive/self-report positive)	14 (58.3%)	10 (41.7%)
Group 2 (grading positive/self-report negative)	257 (97.3%)	7 (2.7%)
Group 3 (grading negative/self-report positive)	34 (82.9%)	7 (17.1%)
Group 4 (grading negative/self-report negative)	4321 (97.7%)	78 (1.9%)

Group 1 (n=24): grading-confirmed age-related macular degeneration (AMD) in association with self-reported AMD; Group 2 (n=264): grading-confirmed AMD in the absence of self-reported AMD; Group 3 (n=41): grading-confirmed absence of AMD in association with self-reported AMD; Group 4 (n=4094): grading-confirmed absence of AMD in association with self-reported absence of AMD; n= number of participants. Grading-confirmed AMD, retinal photographs were graded by a certified grader using a modified version of the International Classification and Grading System for AMD; Self-reported AMD, participants were asked whether a doctor had diagnosed them with AMD. Lutein (L) and/or zeaxanthin (Z) and/or meso-zeaxanthin (MZ) supplement use, use of supplements containing either L and/or Z and/or MZ.

Similarly, 9.1% of participants who self-reported a family history of AMD reported the use of a supplement containing at least one of the three macular carotenoids, compared to just 1.4% who did not report a family history of AMD ($p<0.001$, Pearson Chi-Square) (Table 4.10).

Table 4.10: Lutein and/or zeaxanthin and/or meso-zeaxanthin supplement use by self-report of family history of AMD.

	L and/or Z and/or MZ supplement use	
Family history of AMD	**No (%)**	**Yes (%)**
Don't know	417 (98.6%)	6 (1.4%)
No	3694 (98.0%)	75 (2.0%)
Yes	210 (90.9%)	21 (9.1%)

Family history of AMD, participants self-reporting family history of age-related macular degeneration (AMD). Lutein (L) and/or zeaxanthin (Z) and/or meso-zeaxanthin (MZ) supplement use, use of supplements containing either L and/or Z and/or MZ.

The above findings lead us to re-analyse our logistic regression model to include L/Z/MZ supplement use and self-reported AMD. The effect of these extra variables resulted with the family history variable becoming redundant (p=0.724) and, following the removal of the family history variable, the plasma L variable became non-significant (p=0.064) in the logistic regression model (see Table 4.11). Plasma Z was not significantly related to AMD when we carried out similar analyses as above, but replacing plasma L with plasma Z in each equation.

Table 4.11: Summary of logistic regression analysis for the relationship between grading-confirmed AMD and plasma concentrations of lutein, controlling for self-reported AMD, age and L/Z/MZ supplement use**.**

Variable	**β**	**S.E.**	**Wald**	**df**	**Sig.**	**Exp (B)**
Self-reported AMD	1.719	0.293	34.232	1	0	5.579
Plasma lutein	0.702	0.379	3.427	1	0.064	2.017
Age	0.478	0.085	31.357	1	0	1.613
L/Z/MZ supplement use	0.584	0.315	3.436	1	0.064	1.794
Constant	-3.627	0.169	459.271	1	0	0.027

Grading-confirmed AMD, retinal photographs were graded by a certified grader using a modified version of the International Classification and Grading System for age-related macular degeneration (AMD); self-report AMD, participants were asked whether a doctor had diagnosed them with AMD; plasma lutein, concentrations measured by high performance liquid chromatography; L/Z/MZ supplement use, use of foods supplements containing L and/or Z and/or MZ.

4.4 Discussion

The principal and novel finding in this population-based study was that participants who believed that they suffer from AMD (and irrespective of whether this belief was founded or unfounded) exhibit higher plasma concentrations of L than participants who do not believe that they suffer from this condition (again, irrespective of whether this latter belief was justified or unjustified). Our findings have profound implications for epidemiologic studies investigating the prevalence of, and risk factors for, AMD; moreover, our findings also inform the debate regarding the appropriateness of introducing a screening programme for non-advanced AMD.

In this report, plasma concentrations of L in Group 1 (grading positive/self-report positive) were 2.2 times greater than amongst participants in Group 2 (grading positive/self-report negative) and 2.3 times greater than participants in Group 4 (grading negative/self-report negative). Also, plasma concentrations of L in Group 3 (grading negative/self-report positive) were 1.5 times greater than amongst participants in Group 2 (grading positive/self-report negative) and 1.6 times greater than participants in Group 4 (grading negative/self-report negative). Further, our results also strongly suggest that these findings are attributable to greater use of a supplement containing at least one of the three macular carotenoids amongst those who believed (correctly or incorrectly) that they suffered from AMD. With respect to plasma Z concentrations, only Group 1 (grading positive/self-report positive) had significantly higher concentrations of Z when compared to the other groups. One possible explanation for this could be that this was the only group correctly diagnosed by an ophthalmologist of having AMD, with a doctor recommendation to consume a carotenoid supplement (containing all macular

carotenoids, including Z). Importantly, the data reported herein was recorded as part of Wave 1 of TILDA (between 2009 and 2011), a period when supplementation with MP's constituent carotenoids was already in widespread use for the purpose of managing AMD, in spite of the fact that the findings of AREDS 2 were not published until 2013.[69]

Of the 288 participants with grading-confirmed AMD, 264 participants (92%) were unaware that they were afflicted with the condition (Group 2), an unsurprising finding given that patients with non-advanced AMD are typically unaware of their condition because vision is only profoundly affected if and when the disease progresses to the advanced stage (i.e. GA or CNV)[106] and given that 273 participants (95%) with grading-confirmed AMD in this study suffered from the early (non-advanced) form of the condition.[9]

With respect to epidemiologic studies reporting on serum concentrations of L and/or Z, it would appear that some cross-sectional studies may now need to be re-interpreted in light of our novel findings. For example, reference values for plasma carotenoid published after 1999 may now need to be revisited,[107] especially given that contemporaneous reference values are particularly important because of legitimate concerns that the nutrient content of many fruits and vegetables is reported to have diminished since the introduction of intensive farming.[108-110] One could exclude participants with AMD, participants who believe that they suffer from AMD, participants with a family history of AMD and also participants who use a supplement containing at least one of the three macular carotenoids, for the purposes of generating reference values, but this measure would necessarily render the sample non-representative of the population at large and would exclude a sub-population that is of

particular interest. Another concern arising from our findings rests on the interpretation of cross-sectional epidemiologic studies attempting to investigate a possible association between MP's constituent carotenoids and the prevalence of AMD. Such studies are, in any case, inherently problematic, not least because MP's constituent carotenoids are intracellular compounds and AMD (whether non-advanced or advanced) results in loss of photoreceptors and their axons.[111, 112] In other words, the principal shortcoming of cross-sectional studies in this respect rests not only on the impossibility of determining causality, but also because it is very likely that AMD causes loss of "housing" to accommodate MP (and, therefore, a lack of MP in association with the disease is probably the result [and not the cause] of the disease);[113] accordingly, cross-sectional studies investigating possible relationships between AMD and MP are subject to greater confounding than those investigating possible relationships between AMD and serum concentrations of MP's constituent carotenoids.

Nevertheless, and with full appreciation of the limitations of associative studies, there have been no less than 12 cross-sectional reports attempting to investigate the relationship between serum concentrations of MP's constituent carotenoids and the risk for AMD (see Table 4.12).[14, 38, 53, 102, 103, 114-120] Further, and notwithstanding the fact that many of these cross-sectional studies were performed in the pre-AREDS 2 era, it should be appreciated that lutein-containing supplements were commercially available since 1999,[121] and since that date their use grew substantially as a result of widespread dissemination of their putative benefits.[122-126] Meaningful comment on any such relationship should be predicated, therefore, on population-based studies where data was recorded pre-1999 and to subsequent population-based studies where the use of

carotenoid-containing supplements was recorded and appropriately factored into analyses. Three[38, 53, 102] of four[14, 38, 53, 102] (75%) population-based studies using data recorded pre-1999 found an inverse relationship between AMD and serum concentrations of L and/or Z, and this compares with none of one[118] (0%) population-based studies utilizing data recorded after 1999 where supplement use was recorded and factored into analyses (see Table 4.12).

However, we know that during the period of the TILDA study (Wave 1, 2009 to 2011) eye healthcare professionals in the ROI were actively advising patients on the benefits of carotenoid supplements for AMD (see published survey in Optometry in Practice 2011).[69] Of note, during this time, research from the MPRG, at the Nutrition Research Centre Ireland (a research group based in the south-east of Ireland, at Waterford Institute of Technology; www.nrci.ie) had been reporting on the benefits of carotenoid supplementation for AMD. [36, 127] Indeed, following the positive scientific reports from the MPRG, an Irish TV program (Nationwide) was aired by the main Irish broadcaster (Raidió Teilifís Éireann, RTE) on the 23rd of March 2009. This documentary gained major interest among people with AMD (and their healthcare professionals), as it discussed the potential role of carotenoid supplements for people with this condition. With this in mind, it was decided to investigate the relationships between participant reported family history of AMD, awareness of AMD, and supplement use, in order to test if these relationships explained our observed (and somewhat unexpected) findings.

Table 4.12: Summary of cross-sectional studies designed to investigate a possible relationship between age-related macular degeneration and serum concentrations of macular pigment's constituent carotenoids.

First Author, publication year	Period of data collection	Sample size	Type of study	Correlates under investigation	Use of L and/or, Z and/or MZ containing supplements	Principal finding
EDCC Study Group, 1993[103]	1986-1990	968	C-C	Serum L and Z (combined)	Data not recorded	*Inverse relationship with serum L and Z*
Mares-Perlman *et al*., 1995[114]	1988-1990	334	C-C	Serum L and Z (combined)	Data not recorded	No relationship identified
Mares-Perlman *et al*., 2001[53]	1988-1994	8,222	P-B	Serum L and Z (combined)	Data not recorded	*Inverse relationship with serum L and Z***
Sanders *et al*., 1993[115]		130	C-C	Serum L	Data not recorded	No relationship identified
Moeller *et al*., 2006[14]	1994-98 & 2001-04*|	1,787	P-B	Serum L and Z	Data not factored	No relationship identified
Gale *et al*., 2003[38]	1996-1997	380	P-B	Serum L and Z	Data not recorded	*Inverse relationship with serum Z*
Delcourt *et al*., 2006[102]	1996-1997	640	P-B	Serum L and Z	Data not recorded	*Inverse relationship with serum L and Z*
Dasch *et al*., 2005[116]	2001-2003	910	C-C	Serum L and Z	Data recorded and factored into analyses	No relationship identified
Simonelli *et al*., 2002[117]		94	C-C	Serum L and Z (combined)	Data not recorded	No relationship identified
Michikawa *et a*l., 2009[118]	2005-2006	722	P-B	Serum L and Z (combined)	Data not recorded	No relationship identified.
Cardinault *et al*., 2005[119]		55	C-C	Serum L and Z	Data not recorded	No relationship identified
Zhou *et al*., 2011[120]	2007-2008	263	C-C	Serum L and Z	Data recorded and factored into analyses	*Inverse relationship with serum Z*

Publications organized by publication date in the absence of authors reporting period of data collection; dashed line indicates the year (1999) that lutein-containing supplements were commercially available; principal finding is italicized when an inverse relationship is reported. EDCC Study Group, Eye Disease Case-Control Study Group; Type of study: C-C, case-control study; P-B, population-based study; Correlates under investigation: serum lutein (L) and total zeaxanthin (Z), concentrations measured by high performance liquid chromatography (HPLC); Use of L and/or, Z and/or MZ supplements: Data not recorded; supplement data not collected during study visit; Data not factored into analyses, supplement data was collected but not used in analysis between age-related macular degeneration subjects and control subjects; Data recorded and factored into analyses, data of supplement use was collected during study and factored into analyse, exclusion of subjects consuming supplements, or confirmation that none of the subjects consumed supplements; *Serum concentrations of L and Z were collected in 1994-1998, however data on supplement use was collected 2001-2004; **inverse relationship, though marginal.

The findings reported herein also have profound implications for the debate regarding the appropriateness of introducing a screening programme for non-advanced AMD.[128] A screening programme can only be justified when there is a proven intervention for subjects with pre-disease or asymptomatic disease, and who are identified by use of a test that is sensitive (i.e. few false negatives) and specific (i.e. few false positives).[129] Accordingly, non-advanced AMD would appear to be an ideal candidate for a screening program, especially given our findings that awareness of the condition is associated with supplement use and consequentially increased plasma concentrations of L. Indeed, a screening programme for non-advanced AMD could avail of existing infrastructures (community based cameras, centralised reading centre, etc.), which has been shown to be efficacious and cost-effective for the purposes of screening for diabetic retinopathy[130] (although it would need to be extended to the entire population aged 50 years and older, which represents a substantially greater number of participants than the diabetic population).

The economic argument for screening programmes for non-advanced AMD is not difficult to construct, given that 16% of patients with non-advanced AMD will progress to visually consequential (advanced) AMD within 5 years, and given that *circa* 3.75% of patients with non-advanced AMD will avoid progression to the advanced form of the condition if consuming appropriate antioxidant supplements.[131] For illustrative purposes (Appendix F), and in the interest of consistency, and by extrapolating our published prevalence data for AMD in the Republic of Ireland,[9] we can estimate that *circa* 12,632 persons (i.e. 16% of 78,950 people in the ROI with early AMD) will have progressed to a visually consequential and advanced form of the condition in the five

years following fundus photography.[131] Of those 78,950 persons, *circa* 72,634 persons (because 92% of AMD afflicted persons were unaware that they suffered from this condition) were not empowered to avail of standard of care and reduce their risk of disease progression and visual loss by appropriate use of antioxidant supplements, a measure which would also have resulted in substantial financial savings in the management of 2,724 persons (i.e. 3.75% of 72,634 persons)[131] arising from the avoidance of costs associated with anti-VEGF therapy in patients who went on to develop neovascular AMD ($24,000 per eye per year)[94] and arising from avoidance of costs associated with blindness for patients who went on to develop atrophic AMD (estimated at €21,289 per patient annum for the ROI).[132]

Further, and beyond risk-reduction for disease progression and visual loss, it is important to emphasise that antioxidant supplements also confer visual benefits in patients with non-advanced AMD in the short- and medium-term, and are therefore not solely aimed at risk reduction of ultimate disease progression.[101] Indeed, in the recent study by Akuffo *et al.* (CREST), antioxidant supplementation in patients with non-advanced AMD over a 24-month period was shown to enhance vision in non-advanced AMD patients (a condition traditionally associated with progressive visual loss), reflected in statistically significant improvements in contrast sensitivity, glare disability, photostress recovery, and reading speed.[101] These improvements are not trivial, and improve quality of life in patients with non-advanced AMD, as well as reducing the risk of adverse and vision-related insults to health (e.g. falls and hip fracture).[133]

Accordingly, establishment of a screening programme would facilitate appropriate disease-retarding,[99] sight-saving[99] and vision-optimising[101] nutritional interventions to be offered to subjects who would otherwise be unaware that they are afflicted with the non-advanced form of AMD, and would likely be justified by the financial savings accruing from early detection of disease.

The principal strengths of this study rest on its large population-based sample size (n=4423). Also, and somewhat uniquely, this cohort represents a racially homogeneous sample (99% were white and Irish born). However, this study also has limitations, including the fact that the TILDA study sample excluded individuals who were institutionalised (e.g. living in nursing homes) and, also, individuals aged 75 years and older were underrepresented in the sample.[39] Moreover, dietary data would have enriched the analysis and interpretation of our data, given that we were studying compounds, which are entirely of dietary origin.

4.5 Conclusion

In conclusion, we reported that a belief that one suffers from AMD (irrespective of whether that belief was founded or unfounded) was associated with supplement use and consequentially higher plasma concentrations of L. This finding represents a hitherto unappreciated confounding variable for interpretation of cross-sectional epidemiologic studies investigating relationship between AMD and MP's constituent carotenoids. This work will be submitted for review in *Ophthalmic Epidemiology*, under the title, "Self-reported and actual prevalence of age-related macular degeneration in a population based sample: implications for research and policy in public health ophthalmology".

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Chapter 5: Conclusions and future consideration

5.1 Conclusion

In view of the putative and proven health and functional benefits of L and Z (two of MP's constituent carotenoids) for eye and brain, this PhD work has studied the circulating plasma concentrations of these nutrients and how they relate to human health characteristics and lifestyle factors. This work was produced from the unique TILDA sample generated from participants from the ROI using baseline data captured between 2009 and 2011.

A major strength of TILDA is the design of its sampling method (i.e. all residential addresses in the ROI had an equal probability of selection and all persons aged ≥50 years in each household were eligible). The advantage of this sampling method is that it captured data from a large and randomly selected sample (n=8,175), with sufficient statistical power to identify moderate relationships. It is important to point out that the reports in this thesis were generated from the baseline data (i.e. wave 1) and therefore represent cross-sectional data only. I would also like to acknowledge that there are important areas mentioned in this thesis that merit further research regarding the macular carotenoids such as their uptake and metabolism in the human body, factors that influence their bioavailability, transportation on lipoprotein, distribution in host tissues (i.e. brain and retina), variants in genes related to their concentrations in plasma and tissue, and host factors such as enzymes that are responsible for their metabolism.

A noteworthy message from this thesis and much of the discussion in this final chapter is the appropriateness of introducing a screening programme for non-advanced AMD in the ROI. The recommendation for or against a screening programme should meet the principles adopted by WHO.[1] Screening for a disease is most effective and beneficial when the patient is asymptomatic or the condition is detected early enough to effectively treat. Therefore, the ability to treat and/or prevent a disease adequately is perhaps just as important as detecting the condition. Of note, enrichment of MP following supplementation with the macular carotenoids has been shown to enhance visual function in diseased[2-4] and non-diseased eyes,[5] and reduce the risk disease progression in patients with AMD.[6] Similar to other developed countries, our findings inform the debate regarding the appropriateness of introducing a screening programme for non-advanced AMD.[7, 8] Of interest, Chan *et al.* reported that screening for AMD could be cost-effective when conducted during a diabetic retinopathy (DR) screening programme.[8]

In the ROI, the Department of Health established a national framework action to improve the health and wellbeing of people. In February 2013, the National Screening Service (part of this action) launched a diabetic retinal screening programme.[9, 10] The Diabetic RetinaScreen programme provides free, regular DR screening to people diagnosed with diabetes (Type 1 or 2), who are aged 12 years and over, who are registered with the programme and live in the ROI. The thirty-minute eye screening appointment takes place in photography and grading centres located across the ROI. During the appointment, dilation drops are administered and two photographs of each eye are taken. The photographs are sent to an expert to review and the results are posted

to the individual and the individual's doctor. Similar to non-advanced AMD, patients with early stages of DR may not have any symptoms until the disease advances. When DR is diagnosed early, treatment is effective at reducing or preventing damage to a patient's sight.[11] In summary, my recommendation would be an evaluation of the feasibility of including retinal screening for AMD in the ROI. This requires an economic evaluation (e.g. cost-benefit analysis), an evaluation of ethical concerns (balance of benefits and harm), a method to target those at risk (i.e. entire population aged 50 years old or older), and a pilot study. However, our initial basic assessment (see below) for introducing such a basic screening program in the ROI for AMD suggests that it would offer major benefit for the patient and for our healthcare system.

With respect to cost-effectiveness, and by extrapolating our published prevalence data for AMD in the ROI,[12] we can estimate that *circa* 12,632 persons (i.e. 16% of 78,950 people in the ROI with early AMD) are likely to progress to the visually consequential and advanced form of the condition in the five years following fundus photography.[13] We estimate *circa* 72,634 persons (i.e. 92% of 78,950 people) were unaware that they suffered from this condition and that progression of AMD could have been prevented in *circa* 2,724 persons in the ROI (i.e. 3.75% of 72,634) in the period of 2011 to 2016 if an appropriate screening programme and recommending appropriate use of antioxidant supplements had been in place at the time.

Green *et al.* have demonstrated that AMD accounts for 30% of total cases of registered blindness in the ROI, and have estimated that the cost per person going blind in ROI is €21,289 per annum.[14] Therefore, and given that we know that appropriate supplementation has the potential to save sight in 2,724 persons (with non-advanced

AMD) in the ROI over the next five-year period, this represents an estimated saving of *circa* €194 million for Ireland (see Appendix F). Although not included in this report, the added cost of screening for AMD is believed to be low and cost-effective;[8] however, careful cost benefit analysis is merited.

The Health Service Execute (HSE) (part of the Department of Health) recently denied the renewal of medications that are not covered under the Medical Card Schemes (e.g. macular carotenoid supplements) (Appendix G). The Medical Card Scheme enables individuals (eligibility assessed by means test) to avail of free public health service and co-payment on prescriptions. 52% of adults aged 50 years and older are in receipt of the Medical Card Scheme.[15] The removal of carotenoid supplements from this scheme will most likely result in individuals ceasing their daily consumption of macular carotenoid supplements, if they have no means to pay for the supplement (30 day supply costs *circa* €20). Also, given the strong scientific evidence that shows how supplementation with the macular carotenoids enhances visual performance in patients with non-advanced AMD (in a disease that normally causes a loss of visual function), it is my view that cessation of these supplements in patients with AMD will negatively impact on their vision, with associated loss of independence and quality of life. Indeed, by constructing this nutrition cost-benefit evaluation model for AMD in Ireland, my supervisors (Professor John Nolan and Professor Stephen Beatty) wrote a letter late 2016 to the Department of Health entitled "The Rationale and Financial Argument for Irish Patients with Age-Related Macular Degeneration (AMD) to Consume Appropriate Nutritional Supplements", but the decision to remove carotenoid supplements from the Medical support scheme has thus far has not been reversed by the Irish Government.

5.2 Epilogue

Lifestyles and behaviours towards nutrition have continued to change over the years and will continue to do so (e.g. fad diets [Atkins], choosing better fats [plant-based], "miracle foods" [chia seeds], and dietary supplements [fish oils and carotenoids]). In recent times, the important link between nutrition and disease has been confirmed from longitudinal studies, with a result that society has become more self-conscious and interested in making healthier choices concerning nutrition. Most people do not consume an optimal amount of carotenoids by diet alone. Hence, the recommendation to consume dietary supplements to prevent diseases such as AMD. Therefore, what is the impact of this thesis on our understanding of this relationship?

For the scientific community, I believe this research will contribute to the existing scientific literature, and will hopefully guide future epidemiological studies investigating the relationship between serum concentrations of MP's constituent carotenoids and the risk for AMD on the importance of recording and appropriately factoring the use of carotenoid-containing supplements into analyses. I would also like to acknowledge the abstracts and publications that have utilized the analysis of plasma concentrations of L and Z in the TILDA study. Dr Aisling O'Halloran, postdoctoral research fellow, submitted two abstracts entitled "Circulating biomarkers in older adults with frailty: The Irish Longitudinal Study on Ageing (TILDA)" and "Prevalence of Micronutrient, Inflammatory Stress, Metabolic and Renal Conditions in Frail Older Adults: The Irish Longitudinal Study on Ageing (TILDA)" to the 64th Annual & Scientific Meeting of the Irish Gerontological Society. Dr Joanne Feeney, postdoctoral research fellow, submitted an article entitled "Plasma lutein and zeaxanthin are

associated with better cognitive function across multiple domains in a large population based sample of older adults: The Irish Longitudinal Study on Ageing", which was accepted for publication in the Journal of Gerontology: Medical Sciences.

Also, for society, given that 92% of TILDA participants were unaware that they suffered from AMD and were therefore not empowered to avail of standard of care and reduce their risk of disease progression and visual loss by the appropriate use of antioxidant supplements (especially given our finding that awareness of AMD is indeed associated with supplement usage), it would seem prudent to consider introducing a screening programme for non-advanced AMD.

For myself, my thesis has been a valuable journey, which had many challenges, opportunities and achievements. I have truly enjoyed many aspects of my research including the utilization of the TILDA platform, the resources available at NRCI, the capacity to complete in-house analysis of 5,000 plasma samples, interpretation of a large dataset, experience conducting research studies in humans, as well as personal development through academic publications, project management and collaboration with high profile scientists.

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Publications and presentations

Below, I present my published first author peer-reviewed scientific paper (see Appendix D):

1. **Moran R**, Nolan JM, Stack J, O'Halloran AM, Feeney J, Akuffo KO, Kenny RA, Beatty S. Non-dietary determinants and correlates of plasma lutein and zeaxanthin concentrations in an Irish population (TILDA). Journal of Nutrition, Health and Ageing. 2016:1-8.

Below, I present my scientific paper currently under review:

1. **Moran R**, Beatty S, Stack J, O Halloran AM, Feeney J, Akuffo KO, Peto T, Kenny RA, Nolan JM. Self-reported and actual prevalence of age-related macular degeneration in a population based sample: implications for research and policy in public health ophthalmology. Submitted to Ophthalmic Epidemiology. 2017.

Below, I present my contribution to other published peer-reviewed scientific papers:

1. Feeney J, O'Leary N, **Moran R**, O'Halloran AM, Nolan JM, Beatty S, Young IS, Kenny RA. Plasma Lutein and Zeaxanthin are associated with better cognitive function across multiple domains in a large population-based sample of older adults: The Irish Longitudinal Study on Aging. Submitted to Journals of Gerontology: Medical Sciences. 2017.

2. Ademowo OS, Dias IH, Milic I, Devitt A, **Moran R**, Mulcahy R, Howard AN, Nolan JM, Griffiths H. Phospholipid oxidation and carotenoid supplementation in Alzheimer's disease patients. Submitted to Free Radical Biology and Medicine. 2017.
3. Kelly D, Nolan JM, Howard AN, Stack J, Akuffo KO, **Moran R**, Thurnham DI, Dennison J, Meagher KA, Beatty S. Serum and macular response to carotenoid-enriched egg supplementation in human subjects: The Egg Xanthophyll Intervention clinical Trial (EXIT). Br J Nutr. 2017 Jan 26:1-16.
4. Nolan JM, Power R, Stringham J, Dennison J, Stack J, Kelly D, **Moran R**, Akuffo KO, Corcoran L, Beatty S. Enrichment of Macular Pigment Enhances Contrast Sensitivity in Subjects Free of Retinal Disease: Central Retinal Enrichment Supplementation Trials - Report 1. Invest Ophthalmol Vis Sci. 2016 Jun 1;57(7):3429-39.
5. Akuffo KO, Nolan JM, Stack J, Power R, Kirwan C, **Moran R**, Corcoran L, Owens N, Beatty S. The Impact of Cataract, and Its Surgical Removal, on Measures of Macular Pigment Using the Heidelberg Spectralis HRA+OCT MultiColor Device. Invest Ophthalmol Vis Sci. 2016 May 1;57(6):2552-63.
6. Nolan J.M, Meagher KA., Howard AN, **Moran R**, Thurnham DI, Beatty S. Lutein, zeaxanthin and meso-zeaxanthin content of eggs laid by hens supplemented with free and esterified xanthophylls. Journal of Nutritional Science. 2016; 5.
7. Kelly D, Coen RF, Akuffo KO, Beatty S, Dennison J, **Moran R**, Stack J, Howard AN, Mulcahy R, Nolan JM. Cognitive Function and Its Relationship with Macular Pigment Optical Density and Serum Concentrations of its Constituent Carotenoids. Journal of Alzheimer’s disease. 2015 Aug; 48(1):267-77.

8. Akuffo K.O., Nolan J. M., Stack J., **Moran R**., Feeney J., Kenny R.A, Peto T., O'Halloran A.M., Cronin H. and Beatty S. Prevalence of age-related macular degeneration in the Republic of Ireland. British Journal of Ophthalmology. 2015 Aug; 99(8):1037-44.
9. Nolan JM, Loskutova E, Howard A, Mulcahy R, **Moran R**, Stack J, Bolger M, Coen RF, Dennison J, Akuffo KO, Owens N, Power R, Thurnham D, and Beatty S. The Impact of Supplemental Macular Carotenoids in Alzheimer's disease: A Randomized Clinical Trial. Journal of Alzheimer's disease. 2015; 44(4):1157-69.
10. Akuffo K.O., Nolan J. M., Howard A., **Moran R**., Stack J., Klein R., Klein B., Meur S.M., Sabour-Pickett S., Thurnham D.I., and Beatty S. Sustained supplementation and monitored response with differing carotenoid formulations in early age-related macular degeneration. Eye. 2015 Jul; 29(7):902-12.
11. Nolan J. M., Loskutova E., Howard A., **Moran R**., Mulcahy R., Stack J., Bolger M., Dennison J., Akuffo K.O., Owens N., Thurnham D.I., and Beatty S. Macular Pigment, Visual Function, and Macular Disease among Subjects with Alzheimer's Disease: An Exploratory Study. Journal of Alzheimer's disease. 2014; 42(4):1191-202.

Below, I present my non-peer reviewed publication:

1. **Rachel Moran**. Lutein and Vision. Contribution to the Carotenoid of the Month rubric for the 2nd issue of the EUROCAROTEN newsletter. December 20016.

Below, I present my contributions at scientific conferences in my research field (see Appendices H-L):

1. **Moran R**, Beatty S, Stack J, O Halloran AM, Feeney J, Akuffo KO, Peto T, Kenny RA, Nolan JM. Relationship between plasma concentrations of lutein and zeaxanthin and prevalence (and awareness) of age-related macular degeneration in an older Irish population: The Irish Longitudinal Study on Ageing. 64th Annual & Scientific Meeting of the Irish Gerontological Society 2016. Kerry, Ireland. Poster.
2. Koulman A, Stack J, **Moran R**, Loskutova E, Mulcahy R, Matthews L, Beatty S, Howard AN, Thurnham DI, Griffin JL, Nolan JM. "The assessment of phospholipid as biomarkers of Alzheimer's disease: CARDS study." University Hospital Waterford Research Day 2015. Waterford, Ireland. Oral presentation.
3. **Moran R**, Nolan J, Stack J, O'Halloran AM, Feeney J, Akuffo KO, Kenny RA, and Beatty S. "Non-dietary correlates and determinants of plasma lutein and zeaxanthin concentrations in the Irish Population." Macular Carotenoid Conference 2015. Cambridge, UK, Waterford Institute of Technology Research Day 2015 and Retina 2015. Dublin, Ireland. Poster.
4. **Moran R**, Johnson EJ, Stack J, Akuffo K, Loskutova E, Beatty S, and Nolan JM. "The relationship between dietary intake of lutein and zeaxanthin and their concentration in serum: Introduction of a novel carotenoid dietary screener." International Carotenoid Symposium. July 2014. Park City, Utah, USA. Poster and oral presentation.
5. **Moran R**, Mendes-Pinto MM, Meagher K, Akuffo KO, Beatty S and Nolan JM. "Plasma concentrations of lutein and zeaxanthin, macular pigment, and age-related

macular degeneration: The Irish Longitudinal Study on Ageing (TILDA).” Macular Carotenoid Conference July 2013. Downing College, Cambridge, UK and Retina Conference, Fighting Blindness Ireland. November 2013. Dublin, Ireland.

6. **Moran R**, Mendes-Pinto MM, Meagher K, Akuffo KO, Beatty S and Nolan JM. “The evaluation of carotenoids and antioxidants in human plasma: The Irish Longitudinal Study on Ageing (TILDA)”. TILDA Scientific Advisory Board Meeting April 2013. Trinity College, Dublin, Ireland.

Additional accomplishments and training:

Additional training (see appendices M-Q)

1. Level 3 Phlebotomy Skills (Basic) course. Newcastle, UK. January 2017.
2. Advanced Vision Optometric Tests (AVOT) plus Colour Assessment Diagnosis (CAD) training with John Barbur. Waterford. November 2016.
3. Macular Pigment assessment training for Instituto Nacional de Salud Pública (INSP) technicians. Cuernavaca, Mexico. July 2016.
4. Spectralis OCT+HRA training with Macular Pigment Density Course with Phill Ennion from Heidelberg. Waterford. July 2016.
5. Phospholipid analysis of plasma and red blood cells. Medical Research Council, Human Nutrition Research, Cambridge, England. June 2016.
6. Gas Cylinder Safety Training Workshop. BOC, Cork, Ireland. March 2016.
7. Occupational first aid training FETAC Level 5. Premier Frist Aid Training. August 2013 and October 2015.
8. Biochemistry Laboratory Development: I had the opportunity to take part in the design and layout of two new biochemistry laboratories as well as the ordering/placement of laboratory equipment in Waterford Institute of Technology West Campus, which was required to carry out carotenoid analysis and harvesting of lutein at our research centre. September 2014-February 2015.
9. ReMaT Research Management Training. TuTech, Hamburg, Germany. September 2014.
10. Dietary Assessment Methods Workshop. Nutrition Society, London, UK. March 2014.

11. Advanced Chromatographic Techniques Level 9. Waterford Institute of Technology. October 2013 – January 2014.
12. Age related macular degeneration grader training. Reading Centre, Moorefield's Eye Hospital, London, UK. March 2013.

Memberships

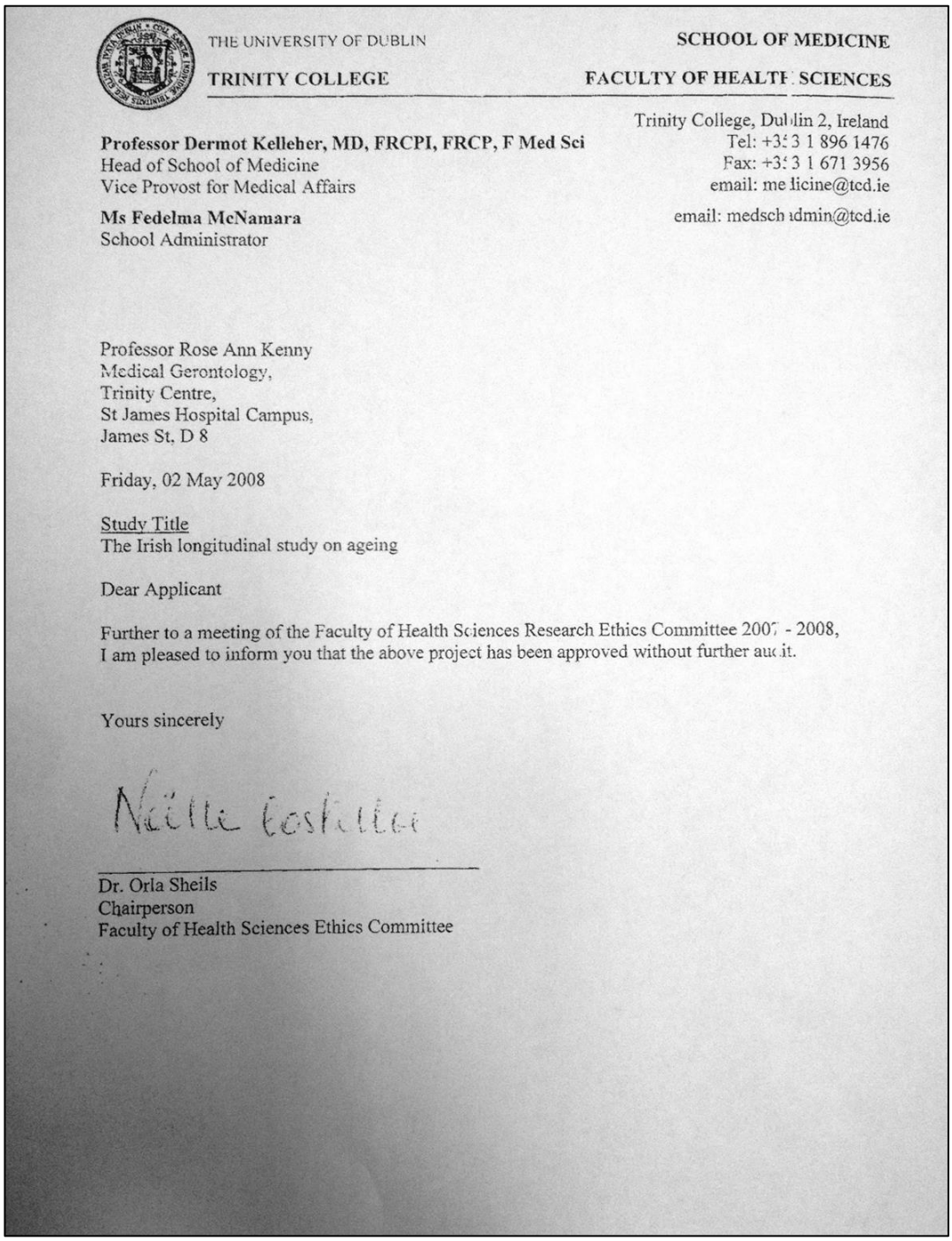
1. Nutrition Research Society. 2014.
2. Think Tank member of the EUROCAROTEN (European Cost action CA15136). 2016-Current.

Scholarships and awards

1. **Third place award for outstanding speaker**. International Carotenoid Symposium. July 2014. (Appendix R)
2. **Presidential Scholarship**. Waterford Institute of Technology. May 2013 – November 2016.

Appendices

Appendix A: TILDA Ethical approval



THE UNIVERSITY OF DUBLIN
TRINITY COLLEGE

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Professor Dermot Kelleher, MD, FRCPI, FRCP, F Med Sci
Head of School of Medicine
Vice Provost for Medical Affairs

Ms Fedelma McNamara
School Administrator
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Professor Rose Ann Kenny
Medical Gerontology,
Trinity Centre,
St James Hospital Campus,
James St, D 8

Friday, 02 May 2008

Study Title
The Irish longitudinal study on ageing

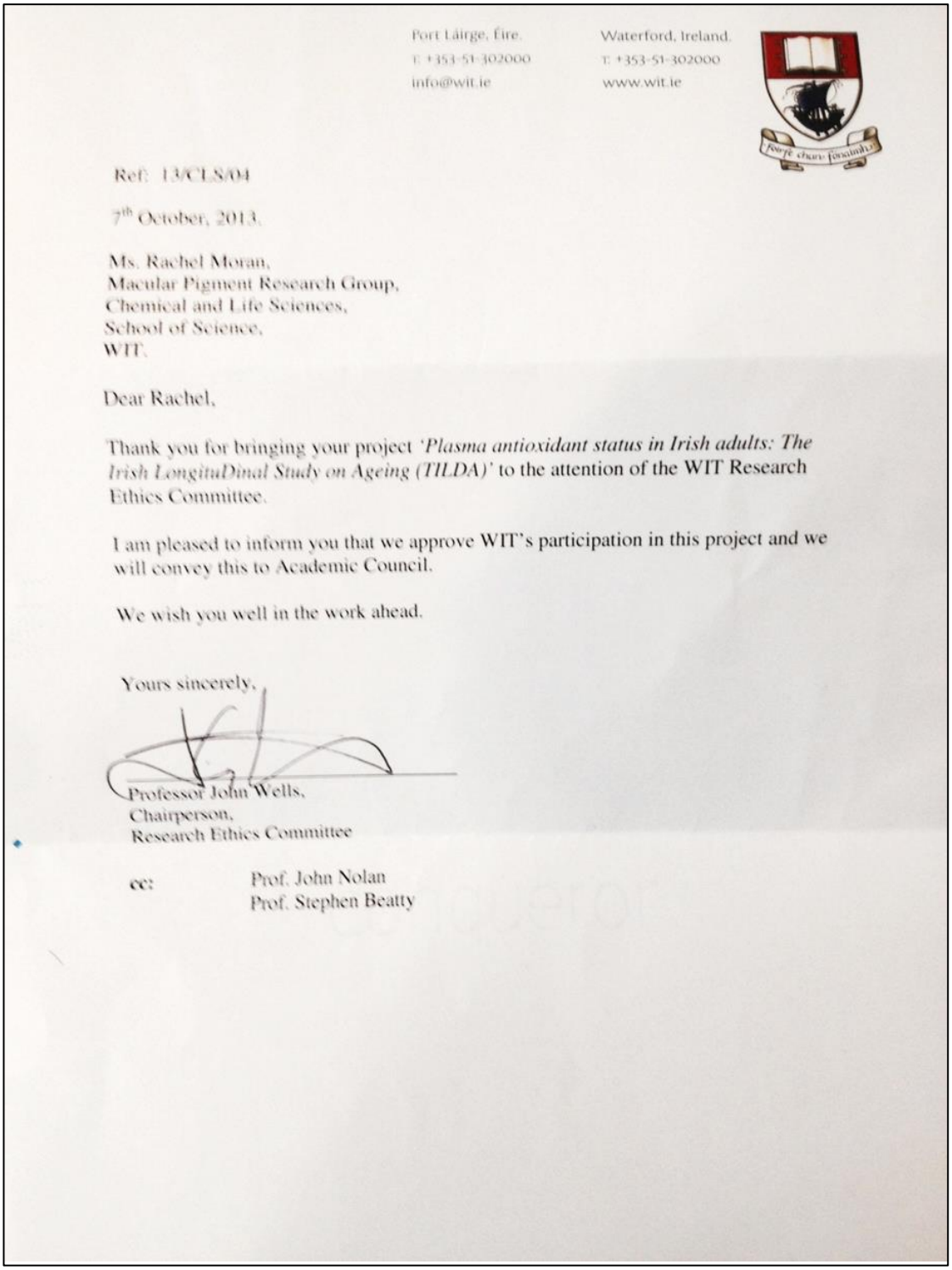
Dear Applicant

Further to a meeting of the Faculty of Health Sciences Research Ethics Committee 2007 - 2008, I am pleased to inform you that the above project has been approved without further audit.

Yours sincerely

Dr. Orla Sheils
Chairperson
Faculty of Health Sciences Ethics Committee

Appendix B: WIT Ethical approval



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Ref: 13/CLS/04

7th October, 2013.

Ms. Rachel Moran,
Macular Pigment Research Group,
Chemical and Life Sciences,
School of Science,
WIT.

Dear Rachel,

Thank you for bringing your project *'Plasma antioxidant status in Irish adults: The Irish LongituDinal Study on Ageing (TILDA)'* to the attention of the WIT Research Ethics Committee.

I am pleased to inform you that we approve WIT's participation in this project and we will convey this to Academic Council.

We wish you well in the work ahead.

Yours sincerely,

Professor John Wells,
Chairperson,
Research Ethics Committee

cc: Prof. John Nolan
Prof. Stephen Beatty

Appendix C: NIST 968e

National Institute of Standards & Technology

Certificate of Analysis

Standard Reference Material® 968e

Fat-Soluble Vitamins, Carotenoids, and Cholesterol in Human Serum

This Standard Reference Material (SRM) is intended for use in validating methods for determining fat-soluble vitamins, carotenoids, and cholesterol in human serum and plasma. This SRM can also be used for quality assurance when assigning values to in-house control materials for these constituents. A unit of SRM 968e consists of three vials, each containing 1 mL of frozen human serum at one of three different concentrations levels.

Certified Values: The certified mass concentration and amount concentration [1] values of retinol, selected tocopherols and carotenoids, cholesterol, and 25-hydroxyvitamin D_3 [25(OH)D_3] in SRM 968e are provided in Table 1. A NIST certified value is a value for which NIST has the highest confidence in its accuracy in that all known or suspected sources of bias have been investigated or taken into account [2]. The certified value for cholesterol was determined from measurements using the NIST reference method, gas chromatography-isotope dilution mass spectrometry (GC-IDMS). The certified value for 25(OH)D_3 was determined from measurements using the NIST reference method, liquid chromatography-isotope dilution tandem mass spectrometry (LC-IDMS/MS). The certified concentration values for the fat-soluble vitamins and carotenoids are based on the agreement of results from two different liquid chromatography (LC) procedures performed at NIST and the median of results from an interlaboratory comparison exercise among institutions that participate in the NIST Micronutrients Measurement Quality Assurance Program. A listing of these institutions is provided in Appendix A.

Reference Values: Reference mass concentration and amount concentration [1] values for additional carotenoids are provided in Table 2. Reference values are noncertified values that are the best estimate of the true values based on available data; however, the values do not meet the NIST criteria for certification and are provided with associated uncertainties that may reflect only measurement precision, may not include all sources of uncertainty, or may reflect a lack of sufficient statistical agreement among multiple analytical methods [2]. The reference values are based on the agreement of results from analytical methods performed at NIST and the median of results from an interlaboratory comparison exercise. Values for some carotenoids are designated as reference values because the identity of components present in the measured chromatographic peak is less certain.

Information Values: Information mass concentration and amount concentration [1] values for additional analytes are provided in Table 3. A NIST information value is considered to be a value that will be of interest to the SRM user, but insufficient information is available to adequately assess the uncertainty associated with the value [2].

Expiration of Certification: The certification of **SRM 968e** is valid, within the measurement uncertainty specified, until **30 April 2020**, provided the SRM is handled and stored in accordance with the instructions given in this certificate (see "Instructions for Storage and Use"). The certification is nullified if the SRM is damaged, contaminated, or otherwise modified.

Maintenance of SRM Certification: NIST will monitor this SRM over the period of its certification. If substantive technical changes occur that affect the certification before the expiration of this certificate, NIST will notify the purchaser. Registration (see attached sheet) will facilitate notification.

The overall direction and coordination of the preparation and analytical measurements leading to the certification of this SRM were performed by J.B. Thomas of the NIST Analytical Chemistry Division.

Analytical measurements at NIST were performed by I.O. Mugenya, L.T. Sniegoski, S.S.-C. Tai, J.B. Thomas, and M.J. Welch of the NIST Analytical Chemistry Division. Collaborating laboratories that performed analyses contributing to value assignment are listed in Appendix A.

Stephen A. Wise, Chief
Analytical Chemistry Division

Gaithersburg, MD 20899
Certificate Issue Date: 12 June 2012
Certificate Revision History on Page 8

Robert L. Watters, Jr., Chief
Measurement Services Division

Statistical consultation was provided by J.H. Yen of the NIST Statistical Engineering Division and D.L. Duewer of the NIST Analytical Chemistry Division.

Support aspects involved in the issuance of this SRM were coordinated through the NIST Measurement Services Division.

NOTICE AND WARNING TO USERS

Warning: SRM 968e IS INTENDED FOR IN-VITRO DIAGNOSTIC USE ONLY. THIS IS A HUMAN-SOURCE MATERIAL. HANDLE PRODUCT AS A BIOHAZARDOUS MATERIAL CAPABLE OF TRANSMITTING INFECTIOUS DISEASE. The supplier of the source materials used to prepare this product found the materials to be non-reactive when tested for hepatitis B surface antigen (HBsAg), human immunodeficiency virus (HIV), hepatitis C virus (HCV), and human immunodeficiency virus 1 antigen (HIV-1Ag) by Food and Drug Administration (FDA) licensed tests. However, because no test method can offer complete assurance that HIV, hepatitis viruses, or other infectious agents are absent, this SRM should be handled at the Biosafety Level 2 for any potentially infectious human serum or blood specimen [3].

INSTRUCTIONS FOR STORAGE AND USE

Storage: Until required for use, SRM 968e should be stored in the dark at or between –20 °C and –80 °C. If carotenoids are to be measured, the unit should be stored at or below –70 °C in the dark. Carotenoids appear to be less stable than the retinoids and the tocopherols at –20 °C [4-7].

Use: SRM 968e is provided as a set of three vials of frozen serum that should be allowed to thaw at room temperature for at least 30 min under subdued light. The contents of a vial should then be gently mixed prior to removal of a test portion for analysis. Precautions should be taken to avoid exposure to strong ultraviolet (UV) light and direct sunlight.

SOURCE, PREPARATION, AND ANALYSIS[1]

Source and Preparation: SRM 968e was prepared from source plasma obtained from Interstate Blood Bank, Inc., Memphis, TN, USA. All units were tested and found negative for HBsAg, HIV, HCV, and HIV-1Ag prior to shipment to NIST. Levels of retinol, γ- and α-tocopherol, and carotenoids were measured at NIST in tubes of plasma obtained from the individual units at the time of plasmapheresis, and blending protocols were specified to result in three materials with different concentration levels. The plasma was shipped by NIST to Solomon Park Research Laboratories (Kirkland, WA, USA) where it was frozen at –80 °C, thawed, and filtered through Whatman 541 filter paper twice to convert it to serum. The serum was pooled, blended, bottled in 1-mL aliquots, and stored at –80 °C prior to shipment back to NIST. Analyte concentrations were *not* adjusted by spiking.

Analytical Approach for Determination of Retinol, Tocopherols, and Carotenoids: The assigned values for selected fat-soluble vitamins and carotenoids in this SRM were derived from results of analyses performed by NIST and 31 collaborating institutions (listed in Appendix A). Because the maintenance of pure and stable primary reference compounds for these analytes is technically difficult, detector responses were calibrated against solutions whose concentrations were determined by spectrophotometry with corrections made for purity as determined by LC. NIST analyses were based on the absorptivities provided in Figure 1. Proteins in the plasma were precipitated with ethanol containing an internal standard as has been previously described [8-10]. Analytes were extracted into hexane, which was evaporated. The reconstituted extracts were then analyzed by liquid chromatography with absorbance detection (LC-UV). Two different LC techniques were used at NIST for the determination of the fat-soluble vitamins and carotenoids in the SRM [8-10]: 1) a polymeric [11] C_{18} column with UV/visible absorbance detection [8,10] and 2) a C_{18} column with different selectivity and absorbance detection [9,10].

Retinol and selected tocopherols and carotenoids were measured in 2 extracts from each of 11 vials of each level of SRM 968e on one day using a 5-µm polymeric [11] C_{18} column (Vydac 201TP, 4.6 mm × 250 mm, Separations Group, Hesperia, CA, USA). A ternary solvent mixture consisting of methanol, butanol, and water was used [8]. UV/visible absorbance detection using a deuterium lamp at the following wavelengths was used: 325 nm for retinol, 292 nm for the tocopherols and tocol (the internal standard), and 450 nm for the carotenoids. This method was also used to assess the homogeneity of the three levels.

---

[1] Certain commercial equipment, instruments or materials are identified in this certificate to adequately specify the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

Retinol and selected tocopherols and carotenoids were measured in two extracts from each of three vials of each level of the SRM on one day using a C_{18} (Bakerbond C_{18} column, 4.6 mm × 250 mm; J.T. Baker, Phillipsburg, NJ, USA) column that exhibits selectivity intermediate to monomeric and polymeric C_{18} columns [11]. A ternary solvent method consisting of acetonitrile, methanol containing 0.05 mol/L ammonium acetate, and ethyl acetate was used [9]. Each of the three solvents contained a volume fraction of 0.05 % triethylamine (TEA) to enhance carotenoid recovery [9]. A programmable UV/visible absorbance detector with a deuterium lamp was used for measurement of retinol, the tocopherols, and the carotenoids at 325 nm, 292 nm, and 450 nm, respectively. *Trans*-β-apo-10'-carotenal oxime [12,13] was used as the internal standard for the quantification of retinol and the carotenoids. Tocol was used as the internal standard for the tocopherols.

Retinol, tocopherols, carotenoids, vitamin K, and coenzyme Q_{10} in SRM 968e were also measured by collaborating institutions that participated in an interlaboratory comparison exercise in which blind samples of the SRM were distributed as part of the NIST Micronutrients Measurement Quality Assurance Program.

Analytical Approach for Determination of 25-Hydroxyvitamin D_3: Concentrations of $25(OH)D_3$ were determined using the NIST LC-IDMS/MS reference method [14]. This method is approved by the Joint Committee for Traceability in Laboratory Medicine (JCTLM) as a higher-order reference measurement procedure [15]. A total of three sets of samples, each set consisting of three to four samples for each of the three levels of SRM 968e were analyzed. Each sample (2 g from combined contents of two vials) was spiked with an isotopically labeled internal standard, 25-hydroxyvitamin D_3-d_3. After equilibration for 1 hr at room temperature, the pH was adjusted with pH 9.8 carbonate buffer, and the sample was extracted with hexane-ethyl acetate (50:50, volume fraction) prior to reversed-phase LC-MS/MS. Atmospheric pressure chemical ionization in the positive ion mode and multiple reaction monitoring (MRM) were used for LC-MS/MS. The transitions at m/z 401 → m/z 383 and m/z 404 → m/z 386 for 25-hydroxyvitamin D_3 and 25-hydroxyvitamin D_3-d_3, respectively, were monitored.

A small amount of 25-hydroxyvitamin D_2 [$25(OH)D_2$] was detected during preliminary measurements for SRM 968e, and an attempt was made to determine the concentrations of $25(OH)D_2$ in SRM 968e using the previously described LC-IDMS/MS method. The limit of quantitation for this method at a signal-to-noise ratio of ≈10 is approximately 0.5 ng/mL. The concentrations of 25-hydroxyvitamin D_2 in SRM 968e were estimated to be below 0.5 ng/mL for all three levels, and therefore were not measured.

Analytical Approach for Determination of Cholesterol: Cholesterol concentrations were determined using the NIST GC-IDMS reference measurement procedure [16,17]. Three sets of samples, each consisting of two vials of each level of the SRM, were analyzed. Two aliquots from each vial were analyzed using an established procedure that employs hydrolysis of cholesterol esters using potassium hydroxide in ethanol, followed by extraction with hexane, and derivatization of cholesterol using *bis*(trimethylsilyl)acetamide [17]. Cholesterol-25,26,27-$^{13}C_3$ was used as the internal standard. Duplicate injections of each sample and each standard were made in each set. Quantitation of cholesterol was achieved by the use of a standard curve obtained by measurement of standards of weighed mixtures of SRM 911c Cholesterol and cholesterol-25,26,27-$^{13}C_3$.

Homogeneity Assessment: The homogeneity of retinol and selected tocopherols and carotenoids was assessed at NIST by using the reversed-phase polymeric C_{18} LC method described above. An analysis of variance did not show inhomogeneity for the test portions analyzed. All measurands were treated as though they were homogeneously distributed, although homogeneity of all measurands was not assessed.

Value Assignment: The equally weighted mean of the two NIST method means and the median of the laboratory means from the interlaboratory comparison exercise were used to calculate certified values for retinol, tocopherols, and carotenoids. The GC-IDMS mean was used to assign certified values for cholesterol. The LC-IDMS/MS mean was used to assign certified values for $25(OH)D_3$. Reference values are based on the median of the laboratory means from the interlaboratory comparison exercise or on the mean of the interlaboratory median with the NIST method means available for that analyte.

Table 1. Certified Values for Selected Fat-Soluble Vitamins, Carotenoids, and Cholesterol in SRM 968e[a]

	Level 1				Level 2				Level 3			
	(µg/mL)		(µmol/L)		(µg/mL)		(µmol/L)		(µg/mL)		(µmol/L)	
Total Retinol	0.341	± 0.016	1.19	± 0.06	0.482	± 0.030	1.68	± 0.10	0.647	± 0.021	2.26	± 0.073
γ-Tocopherol[b]	1.86	± 0.16	4.47	± 0.38	1.432	± 0.081	3.44	± 0.19	2.27	± 0.17	5.45	± 0.41
α-Tocopherol	6.53	± 0.86	15.2	± 2.0	10.33	± 0.14	23.98	± 0.34	19.37	± 0.63	45.0	± 1.5
Total Lutein	0.067	± 0.008	0.117	± 0.014	0.097	± 0.007	0.170	± 0.013	0.124	± 0.010	0.218	± 0.017
Total Zeaxanthin	0.031	± 0.005	0.055	± 0.008	0.029	± 0.006	0.052	± 0.010	0.029	± 0.005	0.052	± 0.009
Total β-Cryptoxanthin	0.041	± 0.006	0.074	± 0.011	0.040	± 0.006	0.072	± 0.011	0.021	± 0.004	0.037	± 0.007
Total β-Carotene	0.099	± 0.018	0.184	± 0.033	0.234	± 0.023	0.436	± 0.042	0.411	± 0.022	0.765	± 0.041
Cholesterol[c]	1467	± 8	3794	± 20	1585	± 8	4099	± 21	1811	± 10	4683	± 25
	(ng/mL)[e]		(nmol/L)[f]		(ng/mL)		(nmol/L)		(ng/mL)		(nmol/L)	
25-Hydroxyvitamin D_3[d]	7.09	± 0.14	17.7	± 0.4	12.9	± 0.3	32.2	± 0.7	19.9	± 0.4	49.6	± 1.0

[a] Each certified concentration value is an equally weighted mean of the means from the two NIST LC methods and the median of the individual laboratory means from the interlaboratory comparison exercise, unless otherwise noted. The results for total retinol include *cis*- plus *trans*-retinol. *Trans*-retinol was not separately determined in the SRM by either method employed at NIST. The uncertainty provided with each value is an expanded uncertainty about the mean to cover the measurand with approximately 95 % confidence: it expresses both the observed difference between the results from the methods and their respective uncertainties, consistent with the ISO Guide and its Supplement 1 [18-20]. The expanded uncertainty is calculated as ku_c, where u_c is the combined uncertainty, and $k = 2$ is a coverage factor corresponding to approximately 95 % confidence for each analyte [18].

[b] Includes β-tocopherol.

[c] The certified concentration value for cholesterol was derived from measurements from three sets of samples using the NIST GC-IDMS method described above. The uncertainty provided with each value is an expanded uncertainty about the mean to cover the measurand with approximately 95 % confidence, consistent with the ISO Guide [18]. The uncertainty incorporates within-method uncertainty and Type B uncertainty components related to the analysis. The expanded uncertainty is calculated as ku_c, where u_c is the combined uncertainty, and $k = 2$ is a coverage factor corresponding to approximately 95 % confidence for each analyte [18].

[d] The certified concentration value for 25-hydroxyvitamin D_3 was derived from measurements using the NIST LC-IDMS/MS method described above. The uncertainty provided with each value is an expanded uncertainty about the mean to cover the measurands with approximately 95 % confidence, consistent with the ISO Guide [18]. The uncertainty incorporates within-method and Type B uncertainty components related to the analysis. The expanded uncertainty is calculated as ku_c, where u_c is the combined uncertainty, and $k = 2$ is a coverage factor corresponding to approximately 95 % confidence for each analyte [18].

[e] Mass concentrations were calculated based using the following measured serum densities: Level 1, 1.02118 g/mL, Level 2, 1.02080 g/mL, and Level 3, 1.02099 g/mL. The uncertainty in the serum density measurements was incorporated in values that are reported relative to units of volume.

[f] Molar concentration levels were calculated from mass concentration levels using the relative molecular mass 400.64 g/mol.

Table 2. Reference Values for Selected Carotenoids in SRM 968e[a]

	Level 1		Level 2		Level 3	
	(µg/mL)	(µmol/L)	(µg/mL)	(µmol/L)	(µg/mL)	(µmol/L)
trans-Lycopene	0.135 ± 0.040	0.252 ± 0.075	0.307 ± 0.039	0.571 ± 0.072	0.49 ± 0.23	0.676 ± 0.070
Total Lycopene	0.234 ± 0.095	0.44 ± 0.18	0.52 ± 0.15	0.97 ± 0.28	0.86 ± 0.17	1.60 ± 0.31
Total α-Carotene	0.011 ± 0.005	0.020 ± 0.009	0.031 ± 0.004	0.058 ± 0.008	0.015 ± 0.002	0.028 ± 0.004
trans-β-Carotene	0.088 ± 0.010	0.164 ± 0.018	0.203 ± 0.020	0.378 ± 0.036	0.363 ± 0.038	0.676 ± 0.070

[a] The reference concentration values are equally weighted means of the means from the two NIST LC/absorbance methods available for that analyte and the medians of the laboratory means from the interlaboratory comparison exercise. The uncertainty provided with each value is an expanded uncertainty about the mean to cover the measurand with approximately 95 % confidence: it expresses both the observed difference between the results from the methods and their respective uncertainties, consistent with the ISO Guide and its Supplement 1 [18-20]. The expanded uncertainty is calculated as ku_c, where u_c is the combined uncertainty, and $k = 2$ is a coverage factor corresponding to approximately 95 % confidence for each analyte [18].

Table 3. Information Values for Additional Compounds SRM 968e[a]

	Level 1		Level 2		Level 3	
	(µg/mL)	(µmol/L)	(µg/mL)	(µmol/L)	(µg/mL)	(µmol/L)
δ-Tocopherol	0.09	0.2	0.07	0.2	0.20	0.5
Total α-Cryptoxanthin	0.016	0.03	0.02	0.04	0.015	0.03
Total *cis*-β-Carotene	0.005	0.009	0.013	0.02	0.016	0.03
Coenzyme Q_{10}	0.9	1.0	1.0	1.1	1.4	1.7
	(ng/mL)	(µmol/L)	(ng/mL)	(µmol/L)	(ng/mL)	(µmol/L)
Phylloquinone (vitamin K_1)	0.4	0.9	0.5	1.1	2.8	6.3

[a] These are noncertified values with no reported uncertainties as there is insufficient information to assess uncertainties [2]. The information values are derived from the median of results reported by fewer than six collaborating laboratories.

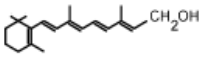
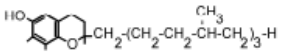
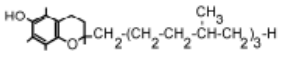
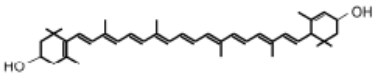
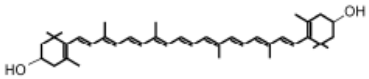
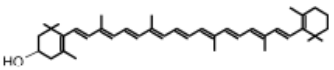
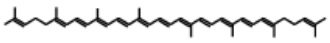
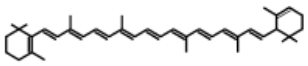
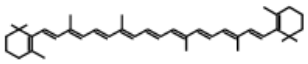
COMPOUND	STRUCTURE	λ_{max}	ABSORPTIVITY
trans-retinol		325 nm	1843 dL/g·cm in ethanol
γ-tocopherol		298 nm	91.4 dL/g·cm in ethanol
α-tocopherol		292 nm	75.8 dL/g·cm in ethanol
trans-lutein		445 nm	2550 dL/g·cm in ethanol
trans-zeaxanthin		452 nm	2540 dL/g·cm in ethanol
trans-β-cryptoxanthin		452 nm	2356 dL/g·cm in ethanol
trans-lycopene		472 nm	3450 dL/g·cm in hexane
trans-α-carotene		444 nm	2800 dL/g·cm in hexane
trans-β-carotene		452 nm	2592 dL/g·cm in hexane

Figure 1. Wavelength maxima and absorptivities used for calibration of retinol, tocopherol, and carotenoid analyses at NIST [21-26].

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Certificate Revision History: 12 June 2012 (Editorial changes); 25 August 2011 (Added certified values for 25-hydroxyvitamin D_3, editorial changes); 30 September 2010 (Original certificate issue date).

Users of this SRM should ensure that the Certificate of Analysis in their possession is current. This can be accomplished by contacting the SRM Program: telephone (301) 975-2200; fax (301) 948-3730; e-mail srminfo@nist.gov; or via the Internet at http://www.nist.gov/srm.

APPENDIX A

Analysts at the institutions listed below performed measurements that contributed to the value assignment of constituents in SRM 968e.

ARUP Laboratories, Salt Lake City, UT, USA
Bio-Reference Laboratories, Elmwood Park, NJ, USA
Cancer Research Center of Hawaii, University of Hawaii at Manoa, Honolulu, HI, USA
Centers for Disease Control and Prevention, Atlanta, GA, USA
Centro Nacional de Alimentación-CENAN, Instituto Nacional de Salud, Lima. Peru
Biochemical Genetics Laboratory, Duke University, Research Triangle Park, NC, USA
Biochemical Genetics Laboratory, Mayo Clinic, Rochester, MN, USA
Biochemical Genetics Laboratory, University of Pittsburgh Medical Center, Pittsburgh, PA, USA
Children's Hospital and Regional Medical Center, Seattle, WA, USA
Children's Hospital National Medical Center, Washington, DC, USA
Départment de Biologie Intégree, Grenoble, France
Department of Human Nutrition, University of Stellenbosch, Tygerberg Campus, Tygerberg, South Africa
Department of Laboratory Medicine and Pathology, University of Alberta Hospital, Alberta, Canada
Department of Nutrition, Harvard School of Public Health, Boston, MA, USA
Division of Nutritional Sciences, University of Illinois at Urbana-Champaign, Urbana, IL, USA
Fred Hutchinson Cancer Research Center, Seattle, WA, USA
Global Central Laboratory, Highland Heights, KY, USA
Harborview Medical Center, University of Washington, Seattle, WA, USA
Human Nutrition Unit, National Institute for Food and Nutritional Research, Rome, Italy
International Centre for Diarrhoeal Diseases Research, Dhaka, Bangladesh
Kronos Science Laboratory, Phoenix, AZ, USA
Laboratoire de Biochimie, Hôpital Purpan, Toulouse, France
MetaMetrix Medical Laboratory, Duluth, GA, USA
MRC Laboratory for Human Nutrition Research, Cambridge, England
Neonatal Nutrition Research Laboratory, University of Louisville, Louisville, KY, USA
Nutrition Research Laboratory, University of California at San Diego, La Jolla, CA, USA
Pediatric CTRC CORE Laboratory, University of Colorado Health Sciences Center, Denver, CO, USA
Quest Diagnostics, Inc., Chantilly, VA, USA
R&D Analytical Research Center, DSM Nutritional Products, Ltd., Kaiseraugst, Switzerland
Rowett Research Institute, Aberdeen, Scotland
Servicio de Bioquímica Clínica, Hospital Universitario Puerta de Hierro, Madrid, Spain

Appendix D: Peer-reviewed scientific publication

J Nutr Health Aging

NON-DIETARY CORRELATES AND DETERMINANTS OF PLASMA LUTEIN AND ZEAXANTHIN CONCENTRATIONS IN THE IRISH POPULATION

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Abstract: *Objective:* To investigate non-dietary correlates and determinants of plasma lutein (L) and zeaxanthin (Z) concentrations in The Irish Longitudinal Study on Ageing (TILDA) sample. *Design:* Cross-sectional study. *Setting:* Community dwelling adults in the Republic of Ireland (ROI). *Participants:* 3,681 participants aged 50 years and older. *Measurements:* TILDA is a nationally representative prospective cohort study of community dwelling adults aged 50 years and over in the ROI. Demographic and health variables were collected during a face-to-face interview carried out in the home (n=8175), and a substantial proportion of these (n=5035; 62%) also attended a study visit in a health assessment centre. Blood samples collected at baseline (wave 1, the subject of the current study), were analysed for plasma concentrations of L and Z by reversed-phase high performance liquid chromatography, and macular pigment (MP) optical density was also measured (using customized heterochromatic flicker photometry). *Results:* After excluding participants with eye disease, data from 3,681 participants were available for analysis. For this group of participants, plasma L and Z were inversely and significantly associated with body mass index (BMI), and were positively and significantly associated with MP, total cholesterol, high-density lipoprotein (HDL) and low-density lipoprotein (LDL) ($p<0.001$, for all). Plasma L and Z were significantly lower in males, current smokers, participants reporting less physical exercise, and participants reporting lower levels of education ($p<0.05$, for all). Plasma L was significantly higher in participants reporting a family history of age-related macular degeneration (AMD) ($p=0.001$), and in the group of ≥75 years old ($p<0.05$). For each of these variables, the significant associations remained after controlling for other potential confounding variables. *Conclusion:* The findings of this large study indicate that plasma concentrations of L and Z were lower in association with indicators of a poor lifestyle (high BMI, tobacco use, and less physical exercise) and in association with lower education, indicating that modifying lifestyle in a positive way is likely to be reflected in higher concentrations of plasma carotenoids, with consequential and putative health benefits.

Introduction

Three carotenoids, lutein (L), zeaxanthin (Z) and meso-zeaxanthin (MZ), accumulate in the macula, where they are collectively referred to as macular pigment (MP) (1, 2). The macula, a specialized area of the retina, is responsible for central and colour vision. The blue light-filtering (3) and the antioxidant properties (4) of MP render this pigment important for optimising visual function in humans. Indeed, MP has been shown to enhance visual function in diseased (5, 6) and non-diseased eyes (7) and reduces the risk of visual loss in, and progression of, age-related macular degeneration (AMD) (8), the leading cause of blindness in adults over the age of 65 (9-12). Also, several epidemiological studies have shown that participants with a high dietary intake of carotenoids (including L and Z) have a lower prevalence of AMD, when compared to participants with a poor dietary intake of carotenoids (13-15).

Recent studies have demonstrated that L and Z are found in the monkey and human brain (16-18), and work by Johnson et al. has shown that brain concentrations of these carotenoids correlate positively with MP levels (19). Also, various groups have reported that MP and serum concentrations of L and Z correlate positively to measures of global cognitive function (20-22), and work from our group has shown that MP and serum concentrations of L and Z are significantly lower in patients with Alzheimer's disease, when compared with age-matched controls (23). Accordingly, there is a need to understand factors that influence the circulating concentrations of these nutrients deemed important for eye and brain health.

Carotenoids are entirely of dietary origin and, as a result, the plasma concentrations of these compounds are dependent on an individual's dietary intake of food containing these nutrients (e.g. leafy greens, vegetables, fruits and eggs) (24). We know that the concentration of L and Z in human plasma varies dramatically between individuals (25). We also know that supplementation with the macular carotenoids (L, Z, and MZ) increases serum concentrations of each respective carotenoid (26, 27) and MP, in different subject populations (e.g. participants free of retinal disease (7), participants with AMD (6), and participants with Alzheimer's disease (28)). However, it has been shown that many other variables (both modifiable and unmodifiable) are likely to influence the concentrations of L and Z in human plasma and host tissues (e.g. retina and adipose tissue (29)). Previous studies suggest that variables

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such as sex, age, BMI, alcohol consumption, smoking status, physical exercise, serum lipids, genetic background, and ethnicity are associated with plasma concentrations of L and Z (30-38). However, not all reports are in agreement, probably due to differences in methodologies and populations studied.

In the current report, we present findings from The Irish Longitudinal Study on Ageing (TILDA). Baseline data on the social, economic, and health status of 8,175 participants aged 50 years and older was collected between 2009 and 2011 from a random sample in the Republic of Ireland (ROI) (39). Given the putative and proven health and functional benefits of L and Z for eye and brain, we hypothesised that it was important to study correlates and determinants of these nutrients in an ageing population. Of note, TILDA allowed us to address this research question, using its large, uniquely homogeneous and randomly selected study sample.

Methods

Study design and sampling

Full details of the design, sampling and methodology of TILDA have been previously reported (39). In summary, a nationally representative sample of community dwelling adults was drawn from the Irish Geodirectory, a current and comprehensive record of all residential addresses in the ROI. Addresses were selected by means of RANSAM (a random sampling design for Ireland) using a three stage process where all household residents aged 50 years or older were eligible to participate (40). Wave 1 (baseline) recruitment had an overall response rate of 62% (n=8175). It is planned that these participants will be interviewed every 2 years and the health assessment repeated every 4 years over a ten year period. As TILDA began in 2009, it has now completed wave 1 and 2, and wave 3 is underway. The focus of the current study was baseline (wave 1) only. Participants were required to provide written informed consent prior to participation in the study. This study was approved by the Faculty of Health Sciences Research Ethics Committee of Trinity College Dublin and the local Ethics committee at the Waterford Institute of Technology. All experimental procedures adhered to the tenets of the Declaration of Helsinki.

Interview/questionnaire

As part of wave 1, participants completed a computer-aided personal interview (CAPI) carried out by a trained social interviewer in the participants' home. The questionnaire collected detailed information on many aspects of the participants' lives (demographics, lifestyle and behaviours), socio-economic status, self-reported health (physical and medical history) and medication use. A list of medications taken on a daily basis (coded using Anatomical Therapeutic Chemical (41)), including food supplements (defined according to the Directive 2002/46/EC of the European Parliament and the Council of the European Union, 10 June 2002) was recorded for each participant. The interview was followed by a self-completion questionnaire, which the participant could complete and return via post. Of note, participants were asked whether a doctor had diagnosed them with the following medical conditions: high blood pressure, AMD (including family history), diabetic maculopathy, diabetic retinopathy, cataracts or glaucoma. It is important to note that self-reported data is susceptible to bias, and this limitation must be taken into account when interpreting results here.

Physical health assessment

Participants were invited to attend a comprehensive health assessment carried out by a team of trained nurses in one of two dedicated health centres in Dublin and Cork or have a modified assessment carried out in their own home (39). Height and weight were measured with Seca™ (Seca Ltd., Birmingham, UK) using a standardized protocol, and body mass index (BMI) was calculated as weight (kg)/height (m^2). According to their BMI, participants were categorized into normal (<25 kg/m^2), overweight (25.1-29.9 kg/m^2) or obese (≥30 kg/m^2). Assessment of MP optical density and retinal photographs for AMD grading were only conducted on the 5,035 participants who attended the health centre (i.e. excluding participants who had home health assessments).

Assessment of Macular Pigment Optical Density

Customized heterochromatic flicker photometry (cHFP), a fast and non-invasive procedure, using Macular Densitometer™ (Macular Metrics Corp., Providence, RI, USA) was used to measure MP at the fovea (0.5° eccentricity) with a reference set at 7° eccentricity (parafoveal reference locus). The eye with the best visual acuity was chosen for MP assessment. A full description of the protocol used for the TILDA study is published elsewhere (42, 43). In summary, the subject is required to achieve isoluminance between a blue light wavelength (absorbed by MP), and a green reference wavelength light (not absorbed by MP). This psychophysical technique is customized for each subject, by optimizing the method, taking into account the subject's age and critical flicker frequency (to allow participants reach their end point in testing with minimal variance) (44).

Retinal photography and AMD grading

TILDA nurses were trained and certified by experts (from the Ocular Epidemiology Reading Centre at the University of Wisconsin, Madison, USA) to take retinal photographs using the NIDEK AFCE-210 non-mydriatic auto-fundus camera, through a non-dilated pupil. Pupils were not dilated as this could influence the results of other health measurements such as gait assessment, which was carried out after retinal photography. Retinal photographs were graded by a trained and certified grader using a modified version of the International Classification and Grading System for AMD under the supervision of Moorfields Eye Hospital Reading Centre,

London, UK (9).

Blood collection and processing

Separate written and verbal consent was required to obtain blood samples from participants. Non fasting venous blood samples were collected into one 5 ml Lithium Heparin tube (BD, Becton, Dickinson Limited, Oxford, UK) for immediate analysis and two 10 ml EDTA (BD, Becton, Dickinson Limited, Oxford, UK) tubes for long term storage. Samples were immediately analysed for lipid profile by a commercial laboratory, which includes total cholesterol (TC), high density lipoprotein (HDL), low density lipoprotein (LDL) and triglyceride. One of the EDTA tubes was immediately protected from direct light. This blood sample was centrifuged and 1 ml of EDTA plasma was dedicated to carotenoid assessment and stored at -80°C until time of analysis.

Plasma L and Z assessment

Each participant had 1 ml of frozen EDTA plasma wrapped in tinfoil transported to the Macular Pigment Research Group, Vision Research Centre, Waterford, Ireland (www.mprg.ie). In 2013, L and total Z was analysed using a reversed phase high performance liquid chromatography (HPLC) method. Details of extraction procedures and HPLC analysis are previously described by our research group (23). Method validation was carried out using 968e Fat-Soluble Vitamins, Carotenoids, and Cholesterol in Human Serum Reference Standard from National Institute of Standards and Technology (NIST) and quality checks were frequently evaluated using control plasma samples. Average coefficients of variation were 4% and 6.8% interassay for L and Z, respectively. The limits of quantification were determined to be 0.0021 µmol/L and 0.0027 µmol/L for L and Z, respectively.

Statistical analysis

The statistical package IBM SPSS Statistics for Windows Version 22.0 was used for analysis. General linear models, with plasma L and Z as dependent variables, were the principal method of analysis. We included a core set of explanatory variables in all linear models, consisting of variables which had been identified in previous studies as being associated with plasma L and Z: age, sex, BMI, highest level of education (primary/none, secondary, and third level), smoking status (never, past or current smoker), and family history of AMD (yes, no and don't know). Controlling for these core variables, we also included the following explanatory variables, one at a time, in the general linear models: geographic location (Dublin city/county, another city or town, and rural), plasma cholesterol (TC, HDL, LDL, and triglyceride), alcohol consumption (number of drinks per week), physical exercise derived from the International Physical Activity Questionnaire-short form (inactive [low], minimally active [medium] and health enhancing physical exercise [high]), food supplement use (number of food supplements taken on a regular basis), and self-reported high blood pressure (yes/no). The 5% level of significance was used throughout, without adjustment for multiple testing.

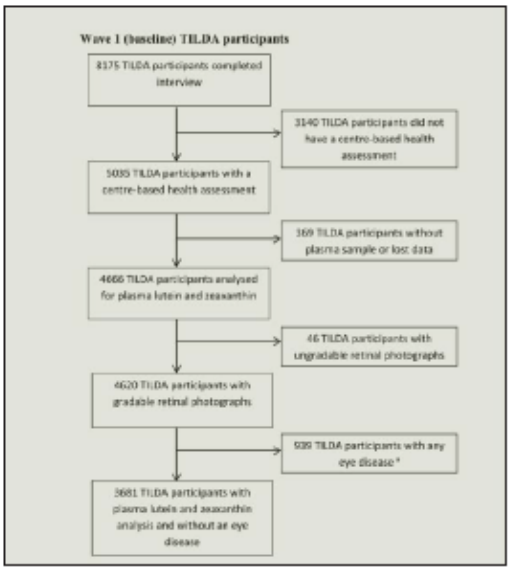
Results

After excluding participants with AMD or self-reported eye disease (diabetic maculopathy, diabetic retinopathy, cataracts or glaucoma), data from 3,681 participants (99% of whom were white, mainly native Irish) were available for analysis (Figure 1). Demographic characteristics for these 3,681 participants studied are reported in Table 1. Of note, the 50-64 age group (70% of our sample) and third level educated respondents (32% of our sample) are over-represented, relative to the population from which the sample was drawn.

Health and lifestyle variables are also presented in Table 1. Mean plasma L and Z concentrations were 0.2047 ± 0.115 µmol/L and 0.0567 ± 0.047 µmol/L, respectively. Plasma concentrations of L and Z were highly correlated with each other; Pearson correlation: r = 0.647, p<0.001. There was also a positive and significant relationship between plasma concentrations of both L and Z, and MP optical density; Pearson correlation: r = 0.242, p<0.001 and r = 0.213 p<0.001, respectively.

Figure 1

The Irish Longitudinal Study on Ageing (TILDA) participants included in this investigation and the time frame for collection and analysis of data; [a] any eye disease includes age-related macular degeneration (AMD) and self-reported: diabetic maculopathy, diabetic retinopathy, cataracts or glaucoma



Relationship of plasma L and Z to core study variables

In the general linear model with putative core explanatory variables (age, sex, MP, education level, BMI, smoking status and family history of AMD), these variables were significantly related to plasma L after controlling for the other variables, with highly significant relationships ($p<0.001$) for sex, BMI, education level, family history of AMD, MP, and smoking status. Most of these core variables were also significantly related to plasma Z ($p<0.001$), the exceptions being age and family history of AMD. Tables 2 and 3 summarise these findings. After the inclusion of food supplement use as a potential explanatory variable, these significant relationships persisted.

Table 1

Demographic, health and lifestyle characteristics of TILDA participants in this investigation

Variable (n=3681)	Mean ± SD or %
Age (years)	60.68 ± 7.70
50-64	70%
65-74	24.4%
75+	5.6%
Sex (Female)	53.1%
Education level	
Primary/none	20.2%
Secondary	42.8%
Third level	37.0%
BMI (kg/m²)	27.45 ± 4.90
Total Cholesterol, mmol/L	5.185 ± 1.050
HDL, mmol/L	1.556 ± 0.436
LDL, mmol/L	2.960 ± 0.940
Smoking status*	
Never/past smoker	84.5%
Current smoker	15.2%
MP Optical Density (0.5°)	0.208 ± 0.159
Plasma L, μmol/L	0.2047 ± 0.115
Plasma Z, μmol/L	0.0567 ± 0.047
Exercise (per week)*	
Low	26.9%
Moderate	35.7%
High	37.4%
Family History of AMD*	5.0%

Data displayed are mean ± standard deviation (SD) for interval data and percentages for categorical data: Variables, variables analysed in the study; n, number of participants; Education level, highest level of education (primary/none, secondary, and third level); BMI, body mass index; Cholesterol, total cholesterol, high density lipoprotein (HDL), and low density lipoprotein (LDL); Smoking status, never-smokers (non-smoker), past smoker and current smoker; MP Optical Density, measured by customized heterochromatic flicker photometry at 0.5°; Plasma L and Z, concentrations measured by high performance liquid chromatography, Exercise, % exercise per week (inactive [low], minimally active [medium] and health enhancing physical exercise [high]); Family history of AMD, % of participants self-reporting family history of age-related macular degeneration; * Self-reported.

As seen in Table 2, females and third level educated participants have significantly higher plasma L and Z concentrations, on average, compared to males and lower educated participants; current smokers have significantly lower average L and Z concentrations compared to past smokers and non-smokers. As seen in Table 3, plasma L and Z concentrations were significantly higher in the low and medium BMI tertile groups when compared to the high BMI tertile group, and were also significantly higher in the medium and high MP tertile groups when compared to the low MP tertile group.

Relationship of plasma L and Z to other study variables

The following potentially confounding variables were then added to the model (which continued to include the core variables), one at a time, with the following statistically significant and positive results: total cholesterol ($p<0.001$, for both plasma L and Z models), HDL ($p<0.001$, for both plasma L and Z models), LDL ($p<0.001$, for both plasma L and Z models). Physical exercise was also significantly related to both plasma concentrations of L and Z, reflected in significantly lower plasma concentrations of these carotenoids amongst participants in the lowest exercise group when compared to the highest exercise group ($p<0.005$). These results are summarised in Tables 2 and 3.

After controlling for the core variables, alcohol consumption was not significantly related to plasma concentrations of L or Z ($p>0.05$, for both), whereas plasma triglycerides were significantly and positively related to plasma concentrations of Z ($p<0.001$) but not to plasma concentrations of L ($p=0.057$), and self-reported hypertension was positively and significantly related to plasma concentrations of Z ($p=0.042$) but not to plasma concentrations of L ($p=0.058$). Use of food supplements was positively related to plasma concentrations of L ($p=0.008$) but not to plasma concentrations of Z ($p=0.059$); of note, after controlling for food supplement use, the significant positive association between plasma L concentration and age remained. Finally, plasma concentrations of L were significantly lower amongst urban dwellers (another city/town, other than Dublin) versus dwellers of rural areas ($p=0.001$).

Discussion

To our knowledge, this is the largest study of its kind to report on the relationships between plasma concentrations of L and Z and non-dietary correlates and determinants of these carotenoids in an older Irish population. The main finding from our study is that plasma concentrations of both L and Z are associated with the following variables: tobacco use, sex, BMI, education, physical exercise, cholesterol status, age (partially), family history of AMD and MP levels. Importantly, the modifiable variables reported here are associated with lifestyle and behavioural habits, and we discuss these findings, and their implications, below.

Table 2
Demographic and lifestyle variables as determinants of plasma lutein and zeaxanthin concentrations

	Mean ± SD[a]					Mean ± SD[a]					Mean ± SD[a]			
	Lutein	Sig.	Zeaxanthin	Sig.		Lutein	Sig.	Zeaxanthin	Sig.		Lutein	Sig.	Zeaxanthin	Sig.
Sex					**Smoking Status**					**Education level**				
Male	0.1869 ± 0.098	<0.001	0.0515 ± 0.038	<0.001	Never	0.2153 ± 0.126	<0.001	0.0607 ± 0.052	<0.001	Primary/none	0.1837 ± 0.106	<0.001	0.0450 ± 0.034	<0.001
Female	0.2203 ± 0.127	-	0.0614 ± 0.053	-	Past	0.2059 ± 0.111	<0.001	0.0563 ± 0.045	<0.001	Secondary	0.1974 ± 0.113	<0.001	0.0553 ± 0.047	<0.001
					Current	0.1690 ± 0.086	-	0.0461 ± 0.031	-	Third/higher	0.2245 ± 0.121	-	0.0649 ± 0.051	-
Age					**Exercise**					**Family history of AMD**				
50-64	0.2051 ± 0.112	0.055	0.0594 ± 0.049	0.299	Low	0.1913 ± 0.105	0.002	0.0517 ± 0.045	0.004	Yes	0.252 ± 0.163	-	0.066 ± 0.004	-
65-74	0.1195 ± 0.110	0.018	0.0495 ± 0.037	0.197	Medium	0.2105 ± 0.120	0.197	0.0579 ± 0.048	0.183	No	0.203 ± 0.113	0.001	0.057 ± 0.0008	0.495
≥ 75	0.2215 ± 0.167	-	0.0554 ± 0.051	-	High	0.2092 ± 0.119	-	0.0594 ± 0.048	-	Don't know	0.194 ± 0.102	<0.001	0.052 ± 0.003	0.074

Data displayed are mean ± standard deviation (SD); a. data expressed as μmol/L, plasma L and Z, concentrations measured by high performance liquid chromatography; Sig, significance difference between groups (dashes indicate reference group e.g. p values for education for comparison of primary and secondary educated subjects with third level educated subjects); smoking status, never-smokers (non-smoker), past smoker and current smoker; exercise, ♀ exercise per week[inactive [low], minimally active [medium] and health enhancing physical exercise [high]]; education level, highest level of education (primary/none, secondary, and third level); family history of AMD, % of participants self-reporting family history of age-related macular degeneration (AMD).

Table 3
Health variables as determinants of plasma lutein and zeaxanthin concentrations

	Plasma lutein concentration (Mean ± SD)[a]				Plasma zeaxanthin concentration (Mean ± SD)[a]			
	1st tertile group	2nd tertile group	3rd tertile group	Sig.	1st tertile group	2nd tertile group	3rd tertile group	Sig.
BMI	0.2344 ± 0.139	0.2038 ± 0.105	0.1755 ± 0.091	<0.001	0.0648 ± 0.055	0.0574 ± 0.047	0.0480 ± 0.035	<0.001
MPOD	0.1770 ± 0.098	0.1970 ± 0.106	0.2379 ± 0.130	<0.001	0.0478 ± 0.035	0.0539 ± 0.044	0.0688 ± 0.056	<0.001
TC	0.1751 ± 0.095	0.2036 ± 0.116	0.2381 ± 0.127	<0.001	0.0453 ± 0.039	0.0554 ± 0.041	0.0706 ± 0.056	<0.001
HDL	0.1690 ± 0.091	0.1993 ± 0.101	0.2478 ± 0.137	<0.001	0.0469 ± 0.043	0.0549 ± 0.043	0.0691 ± 0.051	<0.001
LDL	0.1869 ± 0.112	0.2054 ± 0.113	0.2228 ± 0.118	<0.001	0.0494 ± 0.044	0.0559 ± 0.042	0.0653 ± 0.053	<0.001

Data displayed are mean ± standard deviation (SD); a. data expressed as μmol/L, plasma L and Z, concentrations measured by high performance liquid chromatography; Sig, significance difference between groups; BMI, body mass index (kg/m^2); MPOD, macular pigment optical density 5°; TC, total cholesterol; HDL, high density lipoprotein; LDL, low density lipoprotein.

Firstly, TILDA is a unique longitudinal study, in that it contains data on plasma concentrations of L and Z and MP collected from a large, random, racially homogenous sample of older Irish adults. Secondly, we report here baseline (cross-sectional) data from TILDA, comparing our findings to the findings of other studies including: the Third National Health and Nutrition Examination Survey (NHANES III) (33), the Carotenoids in Age-Related Eye Disease Study (CAREDS) (32), the European Prospective Investigation into Cancer and Nutrition (EPIC) (31), the European Eye study (EUREYE) (30), and some other observational studies (34-37). These studies were conducted between 1988 and 2004, and TILDA (data for this report captured between 2009 and 2011) is much more recent. This is an important point, because some of the factors known to influence circulating concentrations of plasma L and Z have changed in recent years (45,46), such as trends in tobacco use, lifestyle and dietary habits, and use of dietary supplements.

The mean L and Z plasma concentrations reported in our study were comparable with the EUREYE study (30), but were lower when compared to the EPIC study (31). Population and lifestyle differences are likely explanations of these disparities (30, 31).

Consistent with previous reports, we found that tobacco use is associated with significantly lower circulating plasma concentrations of L and Z (33, 34). Current cigarette smokers exhibited 24% and 27% lower plasma concentrations of L and Z, respectively, when compared with non-cigarette smokers. This finding is unsurprising, because cigarette smokers have been shown to have diets lacking in fruits and vegetables (the source of L and Z) (47, 48), but also because it has been shown that cigarette smokers have an increased overall oxidant load, thus reducing circulating plasma carotenoid concentrations (49).

We also found a statistically significant inverse relationship between BMI (a measure of obesity) and plasma concentrations of L and Z, which is also consistent with previous reports (30, 31, 34, 37, 50, 51). Explanations in the literature include an association between BMI and oxidative stress (52), between BMI and diet (53), and competition between adipose tissue and the retina for uptake of the carotenoids from serum (29, 54). Our findings are consistent with the CAREDS study (32), and Kimmons et al. (NHANES III) (55).

Another finding from our study is that plasma L and Z concentrations were positively related to both HDL and LDL. This finding is not surprising, because carotenoids are transported in plasma by these lipoproteins, in a way that relates to the degree of hydrophobicity of those particles (56). However, Clevidence et al. reported that HDL is the primary

carrier of L and Z (57), but our data shows that both HDL and LDL are comparable correlates (and possibly determinants) of plasma concentrations of L and Z, suggesting that L and Z are transported on both lipoproteins. Our findings are consistent with some studies (37, 58, 59) but others report a relationship only with HDL (60-62).

Consistent with the findings reported previously by our group (36, 61), we found in the current TILDA sample that participants reporting a family history of AMD (n=185) had significantly higher plasma L (but not Z) concentrations when compared to participants with no known family history of AMD (n=3496). Importantly, and similar to Nolan et al. (36) and Loane et al. (61), our study compares plasma concentrations of L and Z (separately) for participants with and without a confirmed family history of AMD. In contrast, the CAREDS study reported that a family history of AMD was not significantly related to serum concentrations of L and Z (combined) in elderly women (32). We believe reporting serum concentrations of L and Z (combined) could confound any potential association between serum concentrations of either of these carotenoids (in isolation) and potential correlates and determinants. Our finding that participants with a reported family history of AMD exhibited significantly higher concentrations of L is important, and may reflect carotenoid supplement use in those with a family history of this condition (63). Of note, the ROI is a small country with a population of only 4.6 million (64) with the prevalence of AMD in the population 50 years and older estimated at 7.2% (9). These findings may reflect food supplement use, which is typically more common in the older population, and given that food supplements typically contain high amounts of L (and little or no Z) in their formulations (65). Of note, following extensive media coverage, awareness of AMD in ROI is high, leading to possibly increased supplement use (and consequent higher plasma L) among these 185 participants with family history of AMD. The same explanation may apply to the two older groups (i.e. participants ≥75 years of age having higher plasma L concentrations than those aged 65-74); however, when we controlled for food supplement use in this study, older participants still had significantly higher plasma L concentrations. Our findings are consistent with those of Olmedilla-Alonoso et al., who reported higher serum L concentrations in older adults compared to younger adults, and no association between plasma Z and increasing age (66). Interestingly, O'Connell et al. found that increasing age was associated with reduced dietary intake of Z, but not with reduced dietary intake of L (67).

Also, and consistent with other studies, we found that plasma concentrations of L and Z were higher in female participants when compared to male participants (31, 34, 68), and higher in participants reporting more physical activities (32), which may be explained, at least in part, by better dietary habits in female participants (35, 69) and those performing physical exercise (34, 70). Other correlates of plasma L and Z concentrations in our study include MP and education levels, each of which was significantly and positively related to plasma concentrations of these carotenoids, findings that are consistent with previous reports (32, 71-73). For example, it has been previously shown that plasma L and Z concentrations are important determinants of MP, which is unsurprising given that retinal capture of circulating carotenoids is required to accumulate these nutrients at the macula. Our finding that plasma concentrations of L and Z are related to education levels are consistent with those of Rock et al. and the EUREYE study, which reported that higher serum concentrations of L and Z were associated with third level education (30, 34). Indeed, our findings that education level is a determinant of plasma concentrations of L and Z is also consistent with our previous report from this sample (see Nolan et al. (42)), which found that the level of education was positively and significantly related to MP. Lack of education is associated with many negative correlates (and possibly determinants) of plasma concentration of L and Z including obesity, low physical exercise, tobacco use, and poor diet (74, 75).

The main strengths of this study can be summarised as follows: it is a large (n=3681) randomly selected, racially homogeneous sample (99% white and of Irish birth). Furthermore, standardized methods (including blood processing and storage) and use of a single laboratory to carry out the carotenoid analysis minimized variability. The main limitations of the TILDA study were an underrepresentation of the age group of 75 years and older and underrepresentation of participants who attended primary level education compared with the overall population of ROI (76). Also, dietary data and additional information on food supplement use (ingredients for multivitamins, dose and dose regimen) would have allowed for more detailed analysis with respect to correlates (and putative determinants) of plasma L and Z concentrations in the Irish population.

Conclusion

We report on the demographic, lifestyle and health status of 3,681 mainly white Irish adults over the age of 50 years, in order to investigate correlates and identify potential determinants of plasma concentrations of L and Z. The findings of this large study indicate that plasma concentrations of L and Z are lower in association with indicators of a poor lifestyle (high BMI, tobacco use, and less physical exercise) and lower education, indicating that modifying lifestyle in a positive way is likely to be reflected in higher concentrations of plasma carotenoids with consequential health benefits.

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Conflict of interests: S. Beatty and J.M. Nolan are Directors of Nutrasight Consultancy Ltd, where they do consultancy work for companies with an interest in supplements for eye care. All other authors report no potential conflict of interest.

Ethical Standards: The authors declare that the study procedures comply with the

current ethical standards for investigation involving human participants in the Republic of Ireland. This study was approved by the Faculty of Health Sciences Research Ethics Committee of Trinity College Dublin and the local Institute committee at the Waterford Institute of Technology.

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Appendix E: Chapter 4 Post hoc analysis (Tukey HSD)

Plasma Lutein

Tukey HSD[a,b]

Group	N	Subset for alpha = 0.05		
		1	2	3
4.00	4094	.2040		
2.00	264	.2162		
3.00	41		.3176	
1.00	24			.4691
Sig.		.955	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 57.070.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

Group 1 (n=24): grading-confirmed age-related macular degeneration (AMD) in association with self-reported AMD; Group 2 (n=264): grading-confirmed AMD in the absence of self-reported AMD; Group 3 (n=41): grading-confirmed absence of AMD in association with self-reported AMD; Group 4 (n=4094): grading-confirmed absence of AMD in association with self-reported absence of AMD; n= number of participants; grading-confirmed AMD, retinal photographs were graded by a certified grader using a modified version of the International Classification and Grading System for age-related macular degeneration (AMD); plasma lutein and zeaxanthin expressed as µmol/L, concentrations measured by high performance liquid chromatography.

Plasma Zeaxanthin

Tukey HSD[a,b]

Group	N	Subset for alpha = 0.05	
		1	2
2.00	264	.0551	
4.00	4094	.0557	
3.00	41	.0735	
1.00	24		.1101
Sig.		.149	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 57.070.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

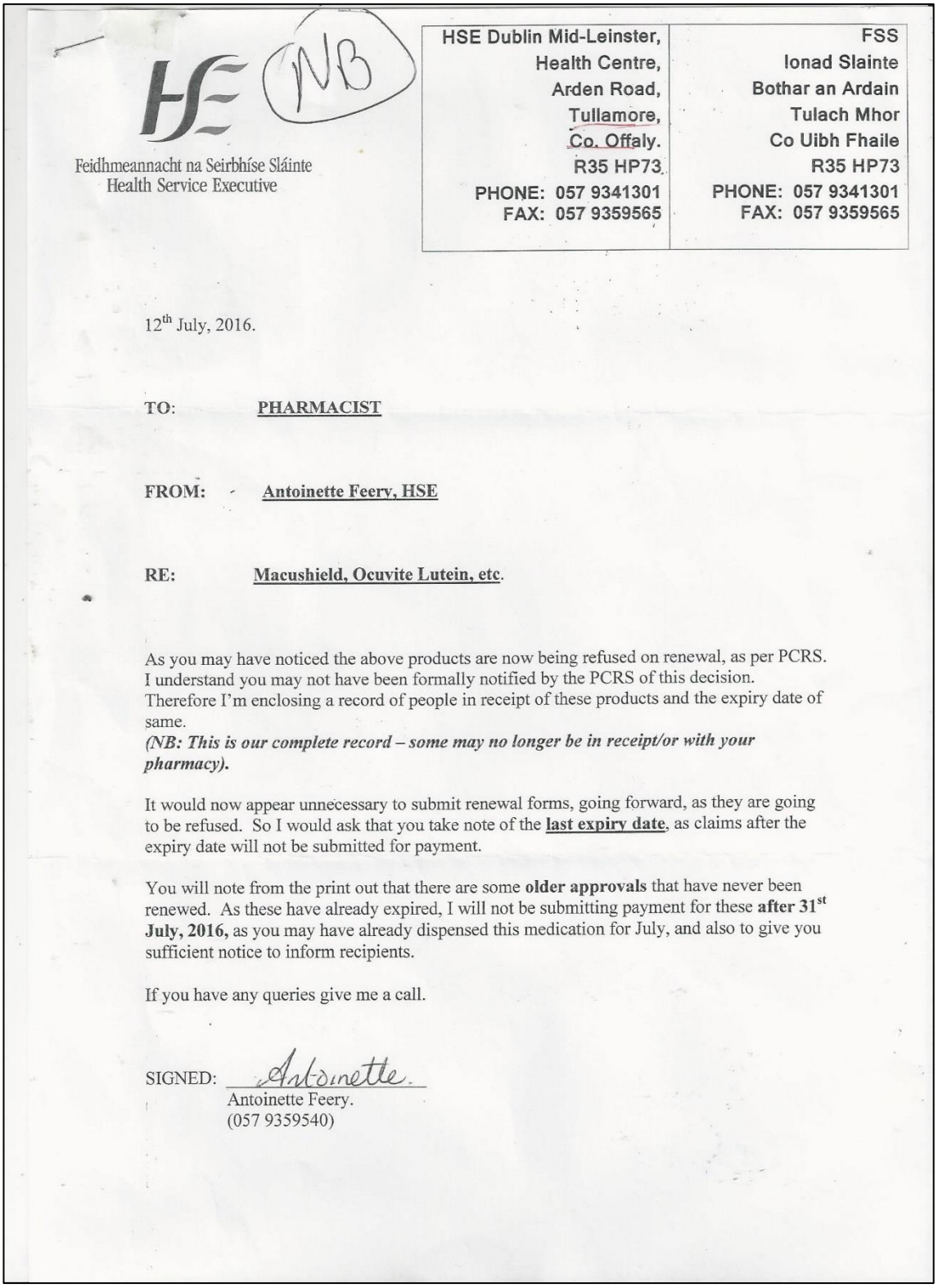
Group 1 (n=24): grading-confirmed age-related macular degeneration (AMD) in association with self-reported AMD; Group 2 (n=264): grading-confirmed AMD in the absence of self-reported AMD; Group 3 (n=41): grading-confirmed absence of AMD in association with self-reported AMD; Group 4 (n=4094): grading-confirmed absence of AMD in association with self-reported absence of AMD; n= number of participants; grading-confirmed AMD, retinal photographs were graded by a certified grader using a modified version of the International Classification and Grading System for age-related macular degeneration (AMD); plasma lutein and zeaxanthin expressed as µmol/L, concentrations measured by high performance liquid chromatography.

Appendix F: Extrapolation of prevalence of age-related macular degeneration (AMD) and costs associated to AMD in Ireland

Code/calculation	Number/cost	Item	Reference
u	**78950**	People in Ireland with early AMD	Akuffo KO, Nolan J, Stack J, et al. Prevalence of age-related macular degeneration in the Republic of Ireland. Br J Ophthalmol 2015;99(8):1037-44.
u*16%=v		People in Ireland will have progressed to advanced AMD within five years (16% of people in Ireland with early AMD)	Bressler NM, Bressler SB, Congdon NG, et al. Potential public health impact of Age-Related Eye Disease Study results: AREDS report no. 11. Arch Ophthalmol 2003;121(11):1621-4.
v	**12632**		
u*92%=w		People in Ireland unaware they are afflicted with early AMD (92% of people with early AMD)	Moran R, Beatty S, Stack J, O Halloran AM, Feeney J, Akuffo KO, Peto T, Kenny RA, Nolan JM. Self-reported and actual prevalence of age-related macular degeneration in a population based sample: implications for research and policy in public health ophthalmology. Submitted to Ophthalmology. 2016.
w	**72634**		
w*3.75%=x		The number of people in Ireland that will avoid advanced AMD if they consume nutritional supplements (3.75%)	Bressler NM, Bressler SB, Congdon NG, et al. Potential public health impact of Age-Related Eye Disease Study results: AREDS report no. 11. Arch Ophthalmol 2003;121(11):1621-4.
x	**2724**		
x*50%=y		The number of people in Ireland that will avoid atrophic AMD (50% of cases with late AMD)	Akuffo KO, Nolan J, Stack J, et al. Prevalence of age-related macular degeneration in the Republic of Ireland. Br J Ophthalmol 2015;99(8):1037-44.
y	**1362**		
x*50%=z		The number of people in Ireland that will avoid neovascular AMD (50% of cases with late AMD)	Akuffo KO, Nolan J, Stack J, et al. Prevalence of age-related macular degeneration in the Republic of Ireland. Br J Ophthalmol 2015;99(8):1037-44.
z	**1362**		

Code/calculation	Number/cost	Item	Reference
a	€21,289	Cost of blindness per person in Ireland (direct and indirect costs) per year	Green D, Ducorroy G, McElnea E, et al. The Cost of Blindness in the Republic of Ireland 2010-2020. J Ophthalmol 2016;2016:4691276.
y*a*5=b		The number of people in Ireland that will avoid atrophic AMD x cost of blindness per person in Ireland (direct and indirect costs) per year x 5 years	
b	€144,966,114.94		
y*20,000*5=c		Cost of managing neovascular AMD = €20,000 (on basis of 8 injections per year) for 5 years per person	
c	€136,188,750.00		
b+c=d		Cost avoided if everyone with early AMD were taking supplements	
d	€281,154,864.94		
20*w*60=e		Monthly cost of supplements (€20 for MacuShield) * number of people with early AMD * 60 months (5 years)	
e	€87,160,800.00		
d-e=f		Savings for Irish government	
f	€193,994,064.94		

Appendix G: Letter from the Health Service Executive



Feidhmeannacht na Seirbhíse Sláinte
Health Service Executive

NB

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12th July, 2016.

TO: **PHARMACIST**

FROM: **Antoinette Feery, HSE**

RE: **Macushield, Ocuvite Lutein, etc.**

As you may have noticed the above products are now being refused on renewal, as per PCRS. I understand you may not have been formally notified by the PCRS of this decision. Therefore I'm enclosing a record of people in receipt of these products and the expiry date of same.
(NB: This is our complete record – some may no longer be in receipt/or with your pharmacy).

It would now appear unnecessary to submit renewal forms, going forward, as they are going to be refused. So I would ask that you take note of the **last expiry date**, as claims after the expiry date will not be submitted for payment.

You will note from the print out that there are some **older approvals** that have never been renewed. As these have already expired, I will not be submitting payment for these **after 31st July, 2016,** as you may have already dispensed this medication for July, and also to give you sufficient notice to inform recipients.

If you have any queries give me a call.

SIGNED: Antoinette.
Antoinette Feery.
(057 9359540)

Appendix H: 64th Annual & Scientific Meeting of the Irish Gerontological Society

Relationship between plasma concentrations of lutein and zeaxanthin and prevalence (and awareness) of age-related macular degeneration in an older Irish population: The Irish Longitudinal Study on Ageing

Rachel Moran,[1] John M. Nolan,[1] Jim Stack,[1] Aisling M. O'Halloran,[2] Joanne Feeney,[2,3] Kwadwo O. Akuffo,[1] Rose Anne Kenny,[2] and Stephen Beatty[1]

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Background

Age-related macular degeneration (AMD) is the leading cause of legal blindness in the older population (1). It is estimated that the overall prevalence of AMD in the Republic of Ireland (ROI) is 7.2% (2). The macula is a specialized area of the retina that is responsible for central and colour vision. The loss of central vision in patients with AMD impacts significantly on quality of life.

The carotenoids, lutein (L), meso-zeaxanthin (MZ) and zeaxanthin (Z), are concentrated in the macula, where they are referred to as macular pigment (MP). MP protects against AMD and optimizes visual function via their optical and antioxidant properties (3,4).

The aim of this study was to investigate the relationship between AMD (and awareness) and plasma concentrations of L and Z, in the Irish Longitudinal Study on Ageing (TILDA). TILDA is a nationally representative prospective cohort study designed to investigate factors that influence healthy ageing. TILDA is studying the social, economic, and health status of Irish adults aged 50 years and older.

Figure 1: Chemical structure of L, MZ and Z.

Methods

Baseline (Wave 1) demographic and health variables were collected during a face-to-face interview carried out in the participant's own home and a health assessment carried out by a trained nurse in a dedicated health centre.

- The participant's awareness of a family history of AMD was recorded.
- The participant's awareness of a doctor's diagnosis of AMD was recorded, referred to as awareness of AMD.
- Supplementation was also assessed and relates to the use of any dietary supplements.
- Blood samples were analyzed for plasma concentrations of L and Z by high performance liquid chromatography.
- Retinal photographs were graded by a certified grader using a modified version of the International Classification and Grading System for AMD, referred to as grading-confirmed AMD.

Figure 2: Health variables collected as part of in this investigation.

Plasma L and Z concentrations from 4863 participants was available for analysis. In this report, we categorized participants into three groups as follows-

Figure 3: Categories of TILDA participants in the investigation.

Results

- Plasma L and Z concentrations were both significantly higher in Group 1, when compared with Group 2 (p=0.003 and p=0.017, respectively) and Group 3 (p<0.0005 and p=0.007, respectively).

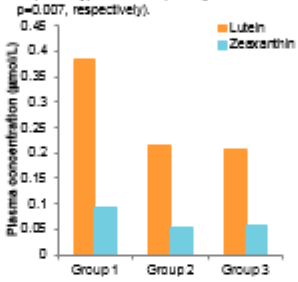

Figure 4: Plasma concentrations of groups.

- Significantly more participants were supplementing in Group 1, compared with Group 2 and Group 3 (p<0.0005).

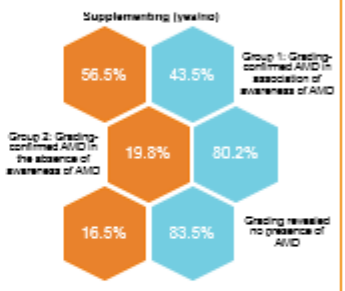

Figure 5: Percentage of groups supplementing.

- Participants with a known positive family history of AMD also had a higher prevalence of the condition (p=0.005), and dietary supplementation was higher in association with a known family history of AMD (p<0.0005).

Figure 6: Family tree.

Figure 7: Risk factors for AMD.

- In the general linear model with core explanatory variables (age, family history of AMD, and supplementation), plasma L was still positively significantly associated with AMD (p=0.003).
- After controlling for core explanatory variables, plasma Z was not associated with AMD (p=0.175).
- Of note, after controlling for the other variables (sex, BMI, education level, family history of AMD, total cholesterol, and smoking status) this significant relationship between plasma L and AMD persisted (p<0.005).

Conclusion

- The findings from this large study indicate that plasma concentrations of L and Z were significantly higher in association with confirmed presence of AMD, awareness of AMD, and supplement use.
- Awareness of family history of AMD was associated with increased usage of dietary supplementation, as well as grading-confirmed AMD. Awareness is relevant in this study, because subjects aware of having AMD, or aware of a family history of this condition, may be more likely to take carotenoid supplements.
- Of the 334 participants with AMD, 264 participants (79%) were unaware they were afflicted with the condition.
- Given the findings of AREDS 2, and the known benefits of supplementation for participants with non-advanced AMD, these results provide a rationale for AMD screening amongst older adults.

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Acknowledgement

We would like to thank Bayer, Ireland (educational grant) and Waterford Institute of Technology (presidential scholarship award) for their support of this study. We would also like to thank the TILDA participants, research team, field researchers and research nurses who conducted tests in TILDA.

Appendix I: Macular Carotenoid Conference 2015, Waterford Institute of Technology Research Day 2015 and Retina 2015

Non-dietary determinants of plasma lutein and zeaxanthin concentrations: results from The Irish Longitudinal Study on Ageing

Rachel Moran[1], Stephen Beatty[1], Jim Stack[1], Aisling M. O'Halloran[2], Joanne Feeney[2], Kwadwo O. Akuffo[1], Rose Anne Kenny[2], and John Nolan[1]

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Macular Carotenoids Conference 2015

Introduction

The three carotenoids, lutein (L), zeaxanthin (Z) and meso-zeaxanthin (MZ), are exclusively found in the central part of the macula, where they are commonly known as macular pigment (MP).

MP has been shown to enhance visual function, and is believed to reduce the risk of age-related macular degeneration (AMD), by absorbing short-wavelength (blue) light, and by neutralising free radicals produced at the macula (from oxidative stress).1

The Irish Longitudinal Study on Ageing (TILDA) was designed to investigate and study factors linked to ageing in the Republic of Ireland. Here, using the unique TILDA study sample, we report on the relationship between the circulating plasma concentrations of L and Z, and non-dietary determinants of these nutrients.

Methodology

Available baseline data on the social, economic, and health status of 5,035 participants aged ≥50 years old in the Republic of Ireland was collected by means of RANSAM sampling (Figure 1).[2]

- Demographic and health variables were collected during a computer assisted personal interview (CAPI) and also at a health assessment.
- Plasma concentrations of L and Z were measured by reversed-phase high performance liquid chromatography (HPLC) .
- MP optical density (MPOD) was measured using customized heterochromatic flicker photometry.

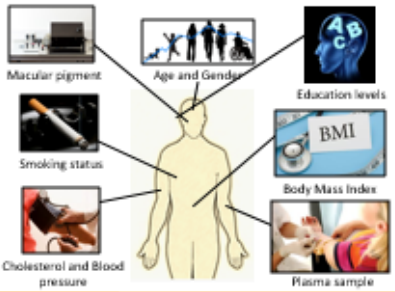

Figure 1: Demographic and health variables collected during baseline assessment.

Results: TILDA Population

After excluding subjects with any eye disease, data from 3,681 participants were available for analysis (Table 1).

Table 1: Demographic, Health, and Lifestyle Variables.

Variable	Mean ± SD or %
Age (years)	60.68 ± 7.70
Sex (Female)	53.1%
Education level	
Primary/none	20.2%
Secondary	42.8%
Third level	37.0%
BMI (Kg/m²)	27.45 ± 4.90
Total Cholesterol, mmol/L	5.185 ± 1.050
HDL, mmol/L	1.556 ± 0.436
LDL, mmol/L	2.960 ± 0.940
Smoking status*	
Never/past smoker	84.8%
Current smoker	15.2%
MP Optical Density (0.5°)	0.208 ± 0.159
Plasma L, µmol/L	0.2047 ± 0.115
Plasma Z, µmol/L	0.0567 ± 0.047
Exercise (per week)*	
Low	26.9%
Moderate	35.7%
High	37.4%
Family history of AMD*	5.0%

SD= Standard deviation, * Self reported

Results: Non-dietary determinants of plasma lutein and zeaxanthin

- Plasma L and Z were significantly lower in males, current smokers, subjects with lower education and subjects reporting less physical exercise ($p<0.05$, Table 2). L was significantly higher in subjects reporting a family history of AMD, and in the group of ≥75 years old ($p<0.05$).
- Plasma concentrations of L and Z were inversely and significantly associated with BMI, and were positively and significantly associated with MP, total cholesterol (TC), high-density lipoprotein (HDL) and low-density lipoprotein (LDL) ($p<0.001$, Table 3).
- For each of these variables in Tables 2 and 3, the significant associations (with L and Z) remained after controlling for the other variables.

Table 2: Determinants of plasma lutein and zeaxanthin concentrations in association with demographic and lifestyle variables.

	Mean ± SD*			Mean ± SD*			Mean ± SD*	
	Lutein	Zeaxanthin		Lutein	Zeaxanthin		Lutein	Zeaxanthin
Gender			**Smoking Status**			**Education**		
Male	0.1869 ± 0.098*	0.0515 ± 0.036*	*Never*	0.2153 ± 0.126	0.0607 ± 0.052	*Primary/none*	0.1837 ± 0.106*	0.0450 ± 0.034*
Female	0.2203 ± 0.127	0.0614 ± 0.053	*Past*	0.2059 ± 0.111	0.0563 ± 0.045	*Secondary*	0.1974 ± 0.113	0.0553 ± 0.047
Age			*Current*	0.1690 ± 0.096*	0.0461 ± 0.031*	*Third/higher*	0.2245 ± 0.121	0.0649 ± 0.051
50-64	0.2051 ± 0.112	0.0594 ± 0.049	**Physical activity**			**Family history of AMD**		
65-74	0.1995 ± 0.110	0.0495 ± 0.037	*Low*	0.1913 ± 0.105*	0.0517 ± 0.045*	*Yes*	0.252 ± 0.162***	0.066 ± 0.004
≥ 75	0.2215 ± 0.167***	0.0554 ± 0.051	*Medium*	0.2105 ± 0.120	0.0579 ± 0.046	*No*	0.203 ± 0.113	0.057 ± 0.0008
			High	0.2092 ± 0.119	0.0594 ± 0.048	*Don't know*	0.194 ± 0.102	0.053 ± 0.003

SD= Standard deviation, *data expressed as (µmol/L, *(p<0.001),** (p<0.05), *** (p<0.001)

Table 3: Determinants of plasma lutein and zeaxanthin concentrations in association with health variables.

	Plasma lutein concentration (Mean ± SD)*				Plasma zeaxanthin concentration (Mean ± SD)*			
	1st tertile group	2nd tertile group	3rd tertile group	Sig.	1st tertile group	2nd tertile group	3rd tertile group	Sig.
BMI	0.2344 ± 0.139	0.2038 ± 0.105	0.1755 ± 0.091	0.000	0.0648 ± 0.055	0.0574 ± 0.047	0.0480 ± 0.035	0.000
MPOD	0.1770 ± 0.098	0.1970 ± 0.106	0.2379 ± 0.130	0.000	0.0478 ± 0.035	0.0539 ± 0.044	0.0688 ± 0.056	0.000
TC	0.1751 ± 0.095	0.2036 ± 0.116	0.2381 ± 0.127	0.000	0.0453 ± 0.039	0.0554 ± 0.041	0.0706 ± 0.056	0.000
HDL	0.1690 ± 0.091	0.1993 ± 0.101	0.2478 ± 0.137	0.000	0.0469 ± 0.043	0.0549 ± 0.043	0.0691 ± 0.051	0.000
LDL	0.1869 ± 0.112	0.2054 ± 0.113	0.2228 ± 0.118	0.000	0.0494 ± 0.044	0.0559 ± 0.042	0.0653 ± 0.053	0.000

SD= Standard deviation, *data expressed as µmol/L , Sig. = statistical significance

Conclusion

- This large study, the first of its kind in Republic of Ireland, mostly confirms the findings from earlier published studies reporting on non-dietary determinants of plasma L and Z.[3,4]
- Importantly, we report that cigarette smoking, the only modifiable established risk factor of AMD, is associated with lack of circulating plasma L and Z, decades before the onset of disease.

Acknowledgements

This work was supported by Bayer, Ireland and the Waterford Institute of Technology Presidential Scholarship. TILDA is funded by the Irish Government, Atlantic Philanthropies and Irish Life plc.

Reference:

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Appendix J: International Carotenoid Symposium 2014

The relationship between dietary intake of Lutein and Zeaxanthin and their concentration in serum: Introduction of a novel carotenoid dietary screener

Rachel Moran,[1] Elizabeth J. Johnson,[2] Jim Stack,[1] Kwadwo Akuffo,[1] Ekaterina Loskutova,[1] Stephen Beatty[1] and John Nolan[1]

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Background

The xanthophyll carotenoids lutein (L) and zeaxanthin (Z) are found in green leafy vegetables (e.g. spinach), colored fruits (e.g. kiwi) and egg yolk.[1] These carotenoids have been studied widely, but mainly because of their likely benefit for macular and cognitive health/function. For example, these carotenoids are believed to protect against age-related macular degeneration (AMD), via their light filtering and antioxidant properties.[2] Also, supplementation with these carotenoids has been shown to enhance visual performance and improve cognitive function.[3, 4]

Given that carotenoids are not synthesized de-novo in humans, their concentration in serum, and target tissues in the body (e.g. retina, brain), is dependent solely on the consumption of food or supplements containing these nutrients. There are many dietary assessment tools available which are used to assess dietary intake of nutrients (such as carotenoids) in epidemiologic and clinical studies (e.g. food frequency questionnaires [FFQs], food diaries and 24-hour recalls), but all these methods have limitations.

For example, they are subjective, and the results are greatly influenced by the accuracy of the portion size reported by the subject. Also, the correctness of the estimates obtained is dependent on the use of accurate nutrient data sources, which will differ greatly between food variety and origin. Finally, these dietary assessment techniques take a long time to complete and are costly, which is not always feasible in a research or clinical setting.

Objective

The motive behind the current study was to investigate if dietary estimates of L and Z obtained using a novel "L/Z screener" is related to serum concentrations of these carotenoids. We see this investigation as an important step in the validation of the "L/Z screener", given that measured serum concentrations of L and Z represent a true biochemical marker of these nutrients.

Figure 1: Examples of natural sources of lutein and zeaxanthin.

Methodology

Data from one hundred and twenty five subjects, obtained from three separate studies conducted at the Macular Pigment Research Group, was used for this investigation. Dietary scores of L and Z (combined) were calculated using the "L/Z screener". Estimates were weighted for frequency of common xanthophyll containing foods and incorporated the frequency of intake, bioavailability and the food xanthophyll content. The range of scores on the "L/Z screener" is 0-75. Serum concentrations of L and Z were assessed by high performance liquid chromatography (HPLC). We investigated, using a general linear model, the relationship between serum L and Z (combined) in these subjects, and their dietary intake as assessed by the "L/Z screener", while controlling for age, smoking status, BMI, sex and study.

Figure 2: Each participant completed a "L/Z screener" and blood samples were obtained for carotenoid analysis.

Results

Subject characteristics are described in Table 1.

Table 1: Demographic, Health, and Lifestyle Variables.

Variable	Mean ± SD
No. of subjects	125
Age (years)	66.3± 9.76
Sex (Female%)	61%
Measured BMI (Kg/m²)	27.36± 3.74
L/Z dietary score	23.85 ± 11.34
Smoking status	
Current Smoker	13
Non smoker/ past smoker	112
LZ serum response (µmol/L)	0.323 ± 0.183

In this study, 33 subjects (26%) exhibited a low dietary carotenoid intake score, 62 subjects (50%) exhibited a medium dietary carotenoid intake score, and 30 subjects (24%) exhibited a high dietary carotenoid intake score (Figure 3).

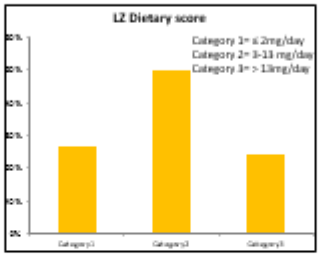

Figure 3: A graph depicting the ranking score which reflects the subjects' relative L/Z intakes.

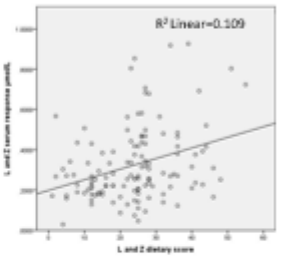

Figure 4: Relationship between L/Z dietary score and serum concentration of L and Z.

Dietary estimates of L and Z combined was positively and significantly correlated with serum concentrations of L and Z combined ($r = 0.329$, $p < 0.001$) (Figure 4), and this relationship remained after controlling for other variables such as age and BMI ($r^2 = 0.216$, $p = 0.003$). However, there remained a considerable amount of unexplained variation in serum concentrations of L and Z when using the "L/Z screener" to predict their concentrations in serum.

Conclusion

- Dietary estimates from the "L/Z screener" may be a useful (albeit crude) indicator of actual serum concentrations of L and Z.
- The "L/Z screener" offers advantages over other dietary assessment tools when estimating L and Z dietary intake, as it is easy to use, quick, and cost effective.
- The "L/Z screener" may be a useful tool to make initial identification of subjects at a certain "L/Z status" for further study.

Acknowledgements

We would like to thank the European Research Council, Howard Foundation and Waterford Institute of Technology (presidential scholarship award) for their support of this study.

References

1. Perry A et al. (2008) Xanthophyll (lutein, zeaxanthin) content in fruits, vegetables and corn and egg products.
2. Sarah Sabour-Pickett et al. (2012) A review of the evidence germane to the putative protective role of the macular carotenoids for age-related macular degeneration.
3. Loughman J et al. (2010) Macular pigment and its contribution to visual performance and experience.
4. Johnson EJ et al. (2013) Relationship between Serum and Brain Carotenoids, alpha-Tocopherol, and Retinol Concentrations and Cognitive Performance in the Oldest Old from the Georgia Centenarian Study.

Appendix K: Macular Carotenoid Conference 2013

Plasma concentrations of lutein and zeaxanthin, macular pigment, and age-related macular degeneration: The Irish LongituDinal Study on Ageing (TILDA)

Rachel Moran, Maria Manuela Mendes-Pinto, Katherine Meagher, Kwadwo Akuffo, Stephen Beatty and John Nolan

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Introduction

The Irish Longitudinal Study on Ageing (TILDA) is a cohort study designed to investigate the factors that influence healthy ageing. TILDA is studying the social, economic, and health status of older Irish adults over a ten year period.

A component of TILDA is investigating the relationship between the plasma concentration of lutein and zeaxanthin, macular pigment (MP) and age-related macular degeneration (AMD).

Sampling

Participants

TILDA recruited a nationally representative sample of 8,175 adults aged 50 years and older.

The sampling frame used was the Irish GeoDirectory, a comprehensive listing and mapping of all residential addresses in the Republic of Ireland compiled by 'an Post' (the Irish Postal Service) and Ordnance Survey Ireland (Figure 1).

Figure 1: Map of selected addresses.

Baseline (Wave 1) data collected

- A face-to-face interview and a self-completion questionnaire which provided information on demographic, lifestyle and health status (Figure 2).
- A total of 5,897 participants agreed to attend a health assessment visit ,where a battery of health data was collected (Figure 3).

Figure 2: (A) Computer assisted personal interviewing (B) self-completion questionnaire.

- Participants recruited at baseline (Wave 1) will have a follow-up interview every two years and a health assessment every four years.

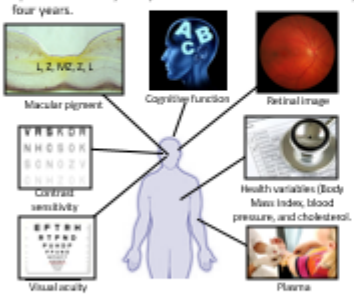

Figure 3: Examples of data collected during health assessment.

Methodology for plasma carotenoid assessment

Extraction

- 0.4 ml of plasma micropipetted into clear eppendorf
- Addition of internal standard (IS), butylated hydroxytolune in ethanol, and Heptane
- Vortexed at highest setting for 2 minutes
- Centrifuged for 5 minutes at 400 g
- 0.4 ml of upper heptane layer removed into amber eppendorf
- Heptane extraction repeated once more
- Dried under nitrogen and stored at -80°C until time of analysis

Figure 4: Carotenoid extraction from human plasma.

Analytical technique

Carotenoids will be analysed by high performance liquid chromatography-reverse phase with photodiode array detector (HPLC-RP-DAD) (Table 1).

Table 1: HPLC conditions.

Column	Phenomenex Ultracarb 3m C18 column (250 x 4.6 mm)
Column Temp.	15°C
Detection	450 nm
Injection volume	100 ml
Mobile phase (MP) A	85% acetonitrile, 15% methanol and 0.1% triethlyamine
Mobile phase B	Dichloromethane
Flow rate	1ml/min
Binary gradient	0-15min (to 10% B); 15-35min (10% B); 35-40min (to 0% B); 40-45min (0% B)
Run time	45 minutes

Qualitative analysis

Lutein and zeaxanthin were identified based on both spectroscopic and chromatographic characteristics (Figure 5 and 6).

- Retention times (Rt)
- Position of the absorption maxima, λ_{max}
- Vibrational fine structure of the UV/Visible spectrum (%III/II)

Quantitative analysis

- External standard calibration
- Parared as IS

Method validation will be made using standards reference 968e from the National Institute of Standards and Technology (NIST).

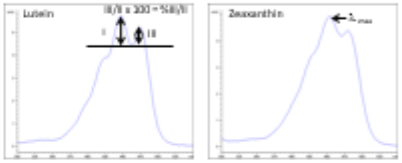

Figure 5: UV/Visible spectrum of L and Z (in mobile phase A).

Carotenoids in Plasma

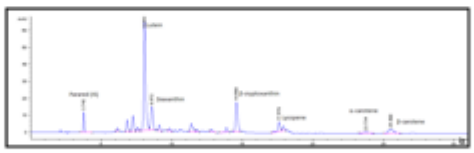

Figure 6: Chromatograph of carotenoids in a plasma sample.

Lutein was identified as the peak that eluted at 8.05 mins, with λ_{max} at 424, 448, 474 nm, and %III/II = 58. Zeaxanthin was identified as the peak that eluted at 8.57 mins, with λ_{max} 428, 452, 478 nm, and %III/II = 17 in. Detection of the following carotenoids β-cryptoxanthin, lycopene, α-carotene, and β-carotene can also be made with this method.

Results

Baseline plasma carotenoid analysis is underway and will be completed by June 2014. MP data has already been analysed and reported [2]. Retinal grading (performed in conjunction with Moorfields Reading Centre, UK) is completed and reporting is underway.

Conclusion

- TILDA provides a uniquely rich opportunity to study, in a large representative sample, the respective relationship between plasma concentration of L and Z, MP and prevalence and incidence of AMD.
- This data will further inform healthcare professionals on the role of the carotenoids, lutein and zeaxanthin, in the pathogenesis of AMD.

Acknowledgement:

We would like to thank Bayer, Ireland (educational grant) and Waterford Institute of Technology (presidential scholarship award) for their support of this study.

Reference:

[1] Britton, G. UV/Visible spectroscopy. 1995. In: *Carotenoids* vol 1B: *spectroscopy* (eds. G. Britton, S. Liaaen-Jensen, H. Pfander), pp. 13-62. Basel, Switzerland.

[2] Nolan JM, Feeney J, Kenny RA, et al. Education is positively associated with macular pigment: The Irish Longitudinal Study on Ageing (TILDA). *Invest Ophthalmol Vis Sci.* 2012;53:7855-7861.

Appendix L: TILDA Scientific Advisory Board Meeting 2013

The evaluation of carotenoids and antioxidants in human plasma: The Irish Longitudinal Study on Ageing (TILDA)

Rachel Moran[1], Maria Manuela Mendes-Pinto[1], Katherine Meagher[1], Stephen Beatty[1,2] and John Nolan[1]

[1]Macular Pigment Research Group, Department of Chemical and Life Sciences, Waterford Institute of Technology, Waterford, Ireland; [2]Institute of Vision Research, Whitfield Clinic, Waterford, Ireland.

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Introduction

The carotenoids lutein, zeaxanthin and meso-zeaxanthin accumulate at the macula where they are known as macular pigment (MP). MP filters short-wavelength blue light, and has powerful antioxidant properties. MP enhances visual performance and reduces the risk of age-related macular degeneration (AMD) via these properties [1]. Given that carotenoids are not synthesized in animals or humans, their concentration in plasma is dependent on intake from diet or supplements (Figure 1). This study will report on the relationship between plasma concentrations of lutein and zeaxanthin and prevalence of AMD in the TILDA sample.

Figure 1: Examples of natural source of lutein and zeaxanthin.

Methodology

Sampling

TILDA has recruited a nationally representative sample of 8,175 adults aged 50 years and older. In Wave 1 (baseline), 5,026 participants have provided a sample of plasma, stored at -80°C until time of analysis.

Extraction

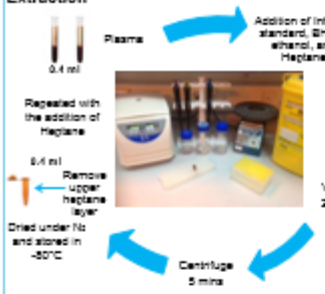

Figure 2: Plasma carotenoid extraction.

Analytical technique

Carotenoids and antioxidants were analysed by high performance liquid chromatography-reverse phase with photodiode array detector (HPLC-RP-DAD) (Table 1).

Table 1: HPLC conditions.

Column	Phenomenex Ultracarb 3µ C18 column (250 x 4.6 mm)
Column temp.	20°C
Detection	450 nm
Flow rate	1ml/min
Injection volume	100 µl
Mobile phase A	85% acetonitrile, 15% methanol and 0.1% triethylamine
Mobile phase B	Dichloromethane
Binary gradient	0-15min (to 10% B); 15-30min (10% B); 30-35min (to 0% B); 35-45min (0% B)
Run time	45 minutes

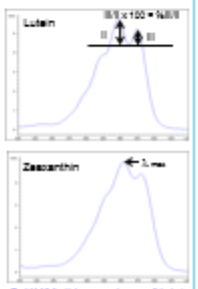

Figure 3: UV/Visible spectrum of lutein and zeaxanthin (in mobile phase A).

Qualitative and Quantitative analysis

Lutein and zeaxanthin were identified based on both chromatographic and spectroscopic characteristics at 450nm (Figure 3 and 4). The retention times (Rt), the positions of the absorption maxima, λ_{max}, and the vibrational fine structure of the UV/Visible spectrum (%III/II), were the parameters used for qualitative analysis of the carotenoids [2], which were compared with those of pure commercial standards.

Quantitative analysis was made using internal standard calibration with Parared as the internal standard (IS). Method validation was made using serum samples of known concentration from the National Institute of Standards and Technology (NIST).

Results

Lutein and Zeaxanthin

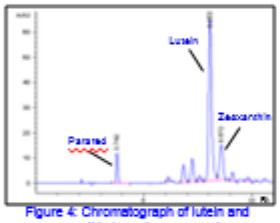

Figure 4: Chromatograph of lutein and zeaxanthin in a plasma sample.

Lutein was identified as the peak that eluted at 8.05 mins, with λ_{max} at 424, 448, 474 nm, and %III/II = 58. Zeaxanthin was identified as the peak that eluted at 8.57 mins, with λ_{max} 428, 452, 478 nm, and %III/II = 17.

Additional carotenoids and antioxidants

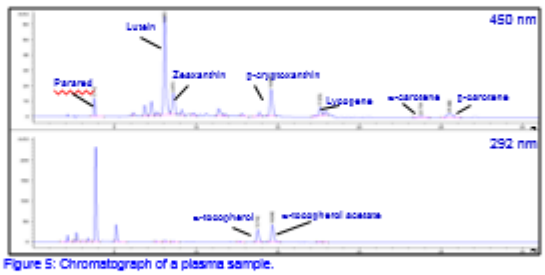

Figure 5: Chromatograph of a plasma sample.

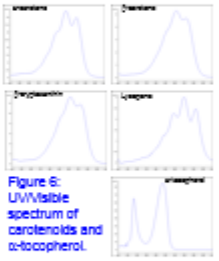

Figure 6: UV/Visible spectrum of carotenoids and α-tocopherol.

This method can also detect the following carotenoids β-cryptoxanthin, lycopene, α-carotene, β-carotene (450 nm) and other antioxidants such as α-tocopherol (vitamin E, at 292 nm using α-tocopherol acetate as IS), as shown in Figure 5 and 6.

Conclusion

This assay provides a sensitive and reproducible method for the analysis of dietary carotenoids and antioxidants in TILDA plasma samples. Along with demographic, lifestyle and behaviour, this data will further inform healthcare on the relationship between the carotenoids lutein and zeaxanthin and AMD, the world's leading cause of age-related blindness.

Acknowledgement:

We would like to thank Bayer, Ireland (educational grant) and Waterford Institute of Technology (presidential scholarship award) for their support of this study.

Reference:

[1] The Age-Related Eye Disease Study 2 (AREDS2) Research Group. Lutein+Zeaxanthin and Omega-3 Fatty acids for Age-Related Macular Degeneration JAMA. 2013 May 5:1-11.
[2] Britton, G. UV/Visible spectroscopy. 1995. In: *Carotenoids vol 1B: spectroscopy* (eds. G. Britton, S. Liaaen-Jensen, H. Pfander), pp. 13-62. Basel, Switzerland; Boston, MA; Berlin, Germany: Birkhäuser.

Appendix M: Dietary Assessment Methods Workshop -Nutrition Society

TRAINING & EDUCATION
Advancing Nutritional Science

Certificate of Attendance

This certificate confirms that

Ms Rachel Moran

attended the

Dietary Assessment Methods workshop on

26 March 2014 at the Nutrition Society Training Rooms

C. Geissler

Professor Catherine Geissler, President of the Nutrition Society

AfN CPD
ENDORSEMENT NO: EN052

Appendix N: ReMaT Research Management Training

TuTech INNOVATION
Wissen . Technologie . Märkte

TEILNAHMEBESTÄTIGUNG / CERTIFICATE OF ATTENDANCE

Wir bestätigen / We certify that

Rachel Moran

die Teilnahme am / has participated in the

Seminar on

Research Management Training Workshop (ReMaT)
16 & 17 September 2014

given by TuTech Innovation, Hamburg

Topics covered included:

- Contexts of modern research
- Managing inter-disciplinary projects
- Invention, Innovation and the Law
- Acquiring research grants in Europe
- Exploiting Research & Technology

Hamburg, 17 September 2014

Monica Schofield
TuTech Innovation GmbH

Margarete Remmert-Rieper
TuTech Innovation GmbH

ReMaT

Appendix O: Training Occupational first aid training FETAC Level 5

FETAC

QQI

Teastas Comhpháirte
Component Certificate

Bronnta ar
Awarded to

Rachel Moran

Level 5 Occupational First Aid Distinction

16/02/2014

Appendix P: Gas Cylinder Safety Training Workshop

BOC
A Member of The Linde Group

CERTIFICATE

This is to certify that

Rachel Moran

of

WIT

attended a Compressed Gases Safety Training course

on

3rd March 2016

and successfully passed the comprehension test on the

SAFE HANDLING AND USE
OF INDUSTRIAL GASES

Damien Adderley
Training Specialist

Adam Jennings-Frisby
Managing Director

Appendix Q: Certificate of Macular Pigment Course

HEIDELBERG ENGINEERING ACADEMY

CERTIFICATE

Spectralis OCT and HRA training with Macular Pigment Density Module Course 5th July 2016

Rachel Moran

successfully completed the **Spectralis OCT and HRA training with Macular Pigment Density Module on 5th July 2016**

Heidelberg Engineering Ltd

Christopher Mody, Director of Clinical Services

05.07.16

Date

Appendix R: International Carotenoid Society Aware 2014