

**Association of dietary and phosphatidylcholine supplement
intake with cardio-metabolic biomarkers among normo- and
hyperlipidaemic individuals.**

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Declaration

I certify that this is my own work and I have acknowledged all material and sources that have been used in this work.

Dedication

To my father, I couldn't have done it without you

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Abstract

Hyperlipidaemia is a worldwide health problem and is associated with the development of cardiovascular disease. Phosphatidylcholine is a component of cellular membranes and plays a vital role in lipoprotein metabolism. Many studies have investigated the effects of phosphatidylcholine on lipid profiles; however, results have been inconsistent, therefore the precise effects of dietary phosphatidylcholine remain unclear.

This project aimed to: i) develop and validate a novel food frequency questionnaire (FFQ) to measure dietary phosphatidylcholine intake; ii) investigate the relationship between dietary phosphatidylcholine intake (as assessed by FFQ) and lipid and cardio-metabolic biomarkers among healthy individuals; and iii) determine the effects of two doses (600 mg and 2400 mg) of phosphatidylcholine supplementation on lipid profiles among hyperlipidaemic individuals.

The study found no significant deviation from linearity ($P = 0.50$) between FFQs and a five-day weighed food record, which indicated that the developed FFQ was reliable and can be used to measure phosphatidylcholine intake in epidemiological studies (Study 1). There was a significant ($P = 0.017$) negative association between dietary phosphatidylcholine intake and triacylglycerol levels (Study 2); however, no relationship was found between dietary phosphatidylcholine intake and other cardio-metabolic biomarkers, including total cholesterol, low density lipoprotein cholesterol, high density lipoprotein cholesterol, and lipoprotein(a) among healthy individuals. Finally, a 2400 mg supplementation study with phosphatidylcholine significantly reduced total cholesterol levels ($P = 0.007$) after a four week intervention, along with a

significant ($P = 0.030$) increase in high density lipoprotein levels in the low dose group (600 mg) of phosphatidylcholine supplementation (Study 3).

In conclusion, these studies have shown that dietary and oral phosphatidylcholine supplementation may play a positive role in normalising lipoprotein metabolism and increasing HDLC, a preventive biomarker of CVD. Further research in the potential of using phosphatidylcholine supplementation as a novel therapeutic agent to treat lipidaemic patients is recommended.

List of abbreviations

Abbreviation	Description
ACAT	Acyl-CoA: cholesterol acyltransferase
AI	Adequate intake
Apo	Apolipoprotein
BMI	Body mass index
CAD	Coronary artery disease
CETP	Cholesteryl ester transfer protein
CVD	Cardiovascular disease
DRVs	Dietary reference values
FFAs	Free fatty acids
FFQ	Food frequency questionnaire
HDLC	High density lipoprotein cholesterol
HTGL	Hepatic triglyceride lipase
IDL	Intermediate density lipoprotein
lysoPLs	lysophospholipids
LCAT	Lecithin–cholesterol acyltransferase
LDLC	Low density lipoprotein cholesterol
LDLr	LDL receptor
Lp(a)	Lipoprotein(a)
LPL	Lipoprotein lipase
mmol	Millimoles per litre
OBU	Oxford Brookes University
PAD	Peripheral arterial disease
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PL	Phospholipids
PS	Phosphatidylserine
PUFA	Polyunsaturated fatty acids
RDA	Recommended dietary allowance
SACN	Scientific Advisory Committee on Nutrition
SD	Standard deviation
SF	Saturated fat
TAGs	Triacylglycerols
TC	Total cholesterol
tHcy	Total homocysteine
TMAO	Trimethylamine N-oxide
UREC	University Research Ethics Committee
VLDL	Very low-density lipoprotein
WR	Weighed food record

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Chapter 1: Introduction and literature review

1.1 Introduction

According to the World Health Organisation (WHO; 2017), cardiovascular disease (CVD) remains the largest cause of death, contributing to 31% of all deaths (approximately 17.9 million people) in 2016 (WHO, 2017). In the United Kingdom (UK), more than 152,000 people died of CVD in 2016 (British Heart Foundation, 2018). In 2013, the National Health Service (NHS) spent approximately £7 billion treating CVD in England alone (Bhatnagar *et al.*, 2015).

CVD includes any disease of the heart or associated blood vessels. The types of CVD affecting blood vessels are: coronary artery disease (CAD), cerebrovascular disease and peripheral arterial disease (PAD). Many risk factors increase the prevalence of CVD including: family history, gender (male), cigarette smoking, diabetes mellitus, excessive use of alcohol, high blood pressure, low levels of serum high density lipoprotein cholesterol (HDL) and high levels of serum low density lipoprotein cholesterol (LDL; Lusis, 2000). However, the build-up of waxy deposits (plaques) on the inner walls of blood vessels is the most common factor in developing heart attacks and strokes (Geovanini and Libby, 2018).

A common CVD is atherosclerosis, which is an inflammatory disease characterised by the aggregation of atherogenic lipids, which leads to the formation of plaques on the interior of artery walls (Lusis, 2000). The disease usually targets large arteries in the body (e.g. the arms, legs or brain; Jacobson *et al.*, 2015). An atherogenic profile is defined by high levels of serum triacylglycerols (TAGs), LDL and Apo B and low levels of serum HDL (Nieves *et al.*, 2003). A level of 4.13 mmol/L or more is defined as a

high LDLC level, whereas a reduction of 1.0 mmol/L can reduce CVD mortality and morbidity by 22% (Baigent *et al.*, 2010). Conversely, high HDL levels can reduce the risk of developing atherosclerosis by promoting a reverse cholesterol pathway, whereby lipid components are removed from the arteries (Libby *et al.*, 2011). A 10% reduction in HDL levels can cause an increase in the risk of CAD by 13% (Després *et al.*, 2000).

The development of dyslipidaemia can be affected by either genetic or/and environmental factors (Catapano *et al.*, 2016). Many drug regimens have been established to treat hyperlipidaemia; one of the most popular are the statin family. Although positive results of statins have been reported in term of treating hyperlipidaemia, many side effects have been recorded such as muscle pain and hyperglycaemia (NHS, 2018). As a result there has been an increased interest in the potential use of nutritional supplements in treating dyslipidaemia (Houston, 2014). One of these supplements is phosphatidylcholine (PC), a phospholipid that plays an important role in cellular membrane structures (Leray, 2014). Studies have explored the effects of PC in different disease states, such as Alzheimer's disease (Whiley *et al.*, 2014), liver disorders (Gundermann *et al.*, 2016) as well as helping normalise lipoprotein circulation in the blood (Høie *et al.*, 2005). However, there is still a large gap in the literature addressing PC supplementation from a nutritional perspective, including the lack of a valid nutritional tool to measure dietary PC intake.

1.2 Lipids: dietary lipids and lipid metabolism

Lipids are water-insoluble molecules, including fats, oils, cholesterol, phospholipids and any other fatty substances. The general structure of lipids includes, carbon, hydrogen, oxygen and phosphorus (only in phospholipids; Gurr *et al.*, 2016).

1.2.1 Types of dietary lipids

Dietary lipids consist of a wide range of lipids comprising polar and non-polar lipids. TAGs are the main lipids in our food and occur at levels of more than 90% above other lipids. The structure of TAGs consists of one molecule of glycerol attached to three fatty acids (Gurr *et al.*, 2016; Figure 1.1).

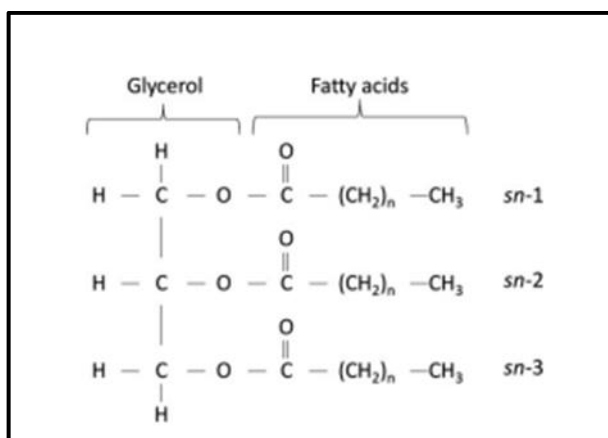


Figure 1.1 The chemical structure of triacylglycerol molecule (Mills *et al.*, 2017)

Fatty acids are traditionally classified as saturated fatty acids, monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), essential fatty acids (including linolenic and linoleic fatty acids), trans-fatty acids and conjugated linoleic acids. Other dietary lipids include, sterols, cholesterol, phospholipids, glycolipids and lipo-soluble vitamins (Leray, 2014).

1.2.2 Recommended intake

Lipids play a vital role in the human body as they are a main source of energy (Gurr *et al.*, 2016). When the body is suffering from starvation or illness, it uses stored fat in adipose tissue as an energy source. Fat storage helps maintain normal body temperature and protects internal organs from injury. In addition, fat plays an important role in the transport and absorption of water-insoluble nutrients (Gurr *et al.*, 2016). For decades the classic advice was to avoid excess dietary fat consumption due

to the link between dietary fat consumption and the risk of CVD, type 2 diabetes and cancers. However, recent evidence shows that consuming less than 30% total fat, as a portion of total energy, was not associated with significant differences in heart disease (Harcombe *et al.*, 2016), cancers (Kim *et al.*, 2006) and diabetes (Salmeron *et al.*, 2001). Researchers now agree that it is less accurate to consider the effects of total fat intake alone on health, whereas contrarily, different types of fats must be considered, for example saturated fat (SF) and trans-fats (Forouhi *et al.*, 2018).

The Scientific Advisory Committee on Nutrition (SACN) has set a dietary fat reference for the UK population, for total fats, as 35% of total daily energy. For SF, MUFA, and PUFA, this should be 10%, 12% and 6% of total daily energy, respectively. Trans-fats should not exceed 2% of the total daily energy (SACN, 2018).

1.2.3 Transport of lipids in the bloodstream

Since lipids are water insoluble they require a carrier for transportation in the bloodstream. These carriers are called lipoproteins.

1.2.3.1 Types of lipoproteins

Although there are several types of lipoproteins that differ in terms of composition and function, their main function is in lipid transportation between tissues (Gurr *et al.*, 2016). Lipoproteins are classified as, chylomicron, very low-density lipoprotein (VLDL), intermediate density lipoprotein (IDL), LDLC, HDLC and Lp(a). The chemical composition of each lipoprotein is represented in Figure 1.2 (Marshall *et al.*, 2016).

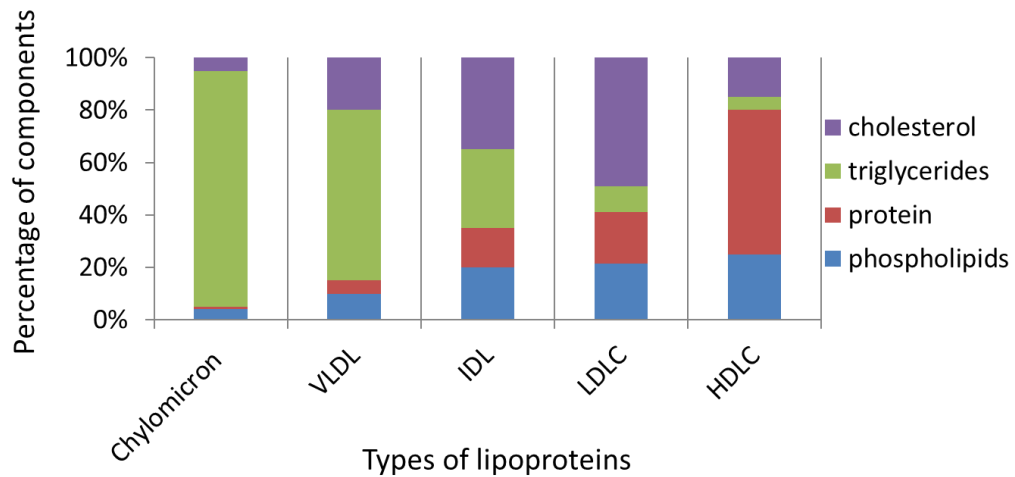


Figure 1.2 Chemical compositions of lipoproteins

Chylomicrons are the least dense lipoprotein. They are rich in TAG particles and are formed in the intestines. The main role of chylomicrons is to transport TAGs and cholesterol to the liver and peripheral tissues. The apolipoproteins AI, AII, AIV, AV, B48, CII, CIII and E are found in chylomicron particles. When TAGs are removed from chylomicrons by peripheral tissues, the chylomicron particles get smaller and become rich in cholesterol, after which they are termed chylomicron remnants (Kingsbury and Bondy, 2003).

VLDLs are also rich in TAGs. These particles are synthesised by the liver as a result of either chylomicron catabolism or TAG synthesis. They contain the apolipoproteins B100, CI, CII, CIII and E; the role of which is to transport lipids to surrounding muscle and adipose tissue. When VLDL particles are absorbed into the muscle and adipose tissue and hydrolysed by the removal of TAGs, IDL particles rich in cholesterol are formed. IDL is taken up from the circulation to the liver via the binding of Apo E to LDLr and the remaining TAGs are hydrolysed by hepatic triglyceride lipases (HTGL). This

process decreases TAGs and exchangeable apolipoproteins are transported from IDL to form LDL (Kingsbury and Bondy, 2003).

LDL particles are rich in cholesterol, as they carry high levels of cholesterol found in the bloodstream. The dominant apolipoprotein found in each LDL particle is Apo B100. LDL is an atherogenic lipoprotein, which means that higher circulating levels of LDL in the blood are related to a higher risk of CVD. The risk of LDL is explained by its main function of carrying cholesterol back to peripheral tissues. LDL particles vary in size depending on the cholesterol quantities they carry. Small dense LDL particles are more harmful than large LDL particles, because they are more sensitive to oxidation, which further promotes the uptake of these particles by macrophages. Similarly, because of their small size, they can easily enter and bind to arterial walls, leading to atherosclerosis (Feingold and Grunfeld, 2018).

The anti-atherogenic lipoprotein HDL is rich in cholesterol and phospholipids and plays a crucial role in the reverse cholesterol pathway that helps transport cholesterol from peripheral tissues back to the liver. HDL contains the apolipoproteins AI, AII, AIV, CI, CII, CIII and E. However, Apo AI is the main protein in the chemical structure of HDL and can be found as multiple molecules in each HDL particle (Marshall *et al.*, 2016). High HDL levels have been shown to reduce the risk of CVD (Libby *et al.*, 2011). The Framingham offspring cohort analysis (Bartlett *et al.*, 2016) of men and women ($n=3590$), without CVD, sought to investigate the risk of CVD in relation to low or high serum HDLC levels (<40 mg/dL in men and <50 mg/dL in women). These were stratified by serum levels of LDLC and TAGs (Bartlett *et al.*, 2016). When compared with an isolated low HDLC, a high HDLC was associated with a 20-40% lower CVD risk. However, most of the studies performed in this area found that after adjustment for

other known risk factors, low HDLC levels were independent predictors of risk. This does not mean, however, that it is causative in CHD.

The mechanisms behind the protective effects of HDL particles in lowering CVD risks are varied. HDL particles promote macrophage cholesterol efflux by enhancing the reverse cholesterol pathway (Rosenson *et al.*, 2012). Similarly, these particles play a role in protecting against inflammation (Barter *et al.*, 2004) and the oxidation of LDL particles (Kontush *et al.*, 2003). In addition, HDL particles promote the maintenance of endothelial function (Oram *et al.*, 1987).

Lipoprotein(a) is a large lipoprotein particle that is rich in cholesterol, phospholipids, TAGs and two other protein molecules; apolipoprotein(a) and apolipoprotein (B100) (Kingsbury and Bondy, 2003).

1.2.3.2 Types of apolipoprotein

Apolipoproteins are a major part of lipoprotein metabolism as they form the main component of lipoproteins and they play crucial roles in the activation/inhibition of enzymes involved in lipoprotein metabolism (Feingold and Grunfeld, 2018). There are several apolipoproteins, but the major human plasma apolipoproteins are apolipoprotein A, apolipoprotein B, apolipoprotein C, apolipoprotein E and apolipoprotein (a) (Irshad and Dubey, 2005).

Apolipoprotein A (Apo AI, AII, AIV, AV): Apo AI is the main protein in HDL, it constitutes approximately 70% of HDL. Apo AI is produced in the liver and intestines. Apo AI activates Lecithin–cholesterol acyltransferase (LCAT) which converts free cholesterol to cholesteryl esters. Apo AII is the second most abundant protein in HDL, at 20% and is synthesised in the liver (Feingold and Grunfeld, 2018). Apo AIV is usually linked with

chylomicrons and HDL particles and is synthesized by the intestines during fat absorption. The function of Apo AIV is not well understood but it is believed to play a role in regulating food intake (Tso *et al.*, 1999). Apo AV is synthesized in the liver and is associated with lipoproteins rich in TAGs, for example chylomicrons. The function of Apo AV is the activation of Lipoprotein lipase (LPL), which is involved in the metabolism of lipoproteins enriched with TAGs (Nilsson *et al.*, 2011).

Apolipoprotein B (Apo B48, Apo B100): Apo B48 is the dominant protein in chylomicrons and chylomicron remnants and is synthesised in the intestines. Apo B100 is synthesised in the liver and is present in VLDL, IDL and LDL particles as a major component. Apo B48 and Apo B100 are found as single molecules in lipoprotein particles (Irshad and Dubey, 2005).

Apolipoproteins C (Apo C, Apo CII, Apo CIII): Apo C proteins are produced in the liver and can be found in chylomicrons, VLDL and HDL. Apo CII is known to activate LPL, which promotes the hydrolysis of TAG particles, thus any mutation in Apo CII function can cause hypertriglyceridemia. Apo CIII inhibits the reaction between TAG-rich lipoproteins and their receptors, studies have shown that a loss in function in Apo CIII can lead to decreases in serum TAGs levels and reductions in CVD risk (Feingold and Grunfeld, 2018).

Apolipoprotein E (Apo E) is primarily synthesised in the liver and intestines but can be produced in other tissues, e.g. nervous tissue. Apo E is part of the following lipoprotein structure: chylomicrons, chylomicron remnants, VLDL, IDL and HDL particles. There are three modified gene subclasses of Apo E (E2, E3 and E4). Apo E has been implicated in CVD and Alzheimer disease (Feingold and Grunfeld, 2018).

Apolipoprotein (a) (Apo (a) is synthesised in the liver and is a unique protein found in Lp(a). Little is known about Apo(a) function, but it is suggested that elevated levels of serum Apo (a) are linked to increased atherosclerosis risks, as the protein blocks the fibrinolysis process which increases the uptake of lipoproteins by macrophages (Feingold and Grunfeld, 2018).

1.2.4 Normal lipoprotein transportation

Lipoprotein metabolism comprises three pathways: 1) exogenous pathway; 2) endogenous pathway; and 3) reverse cholesterol pathway.

The exogenous pathway refers to the transport of dietary TAGs and cholesterol from the intestinal tract to the liver. The process starts when dietary TAGs are hydrolysed by intestinal lipase to free fatty acids and monoacylglycerol and absorbed by small intestine epithelial cells. In these enterocytes, fatty acids and cholesterol become re-esterified and are converted to cholesterol esters and TAGs (Iqbal and Hussain, 2009). After this step, TAGs and cholesterol molecules combine to form nascent chylomicrons that are transported into the bloodstream, through intestinal lymph vessels. When chylomicrons enter the blood circulation, HDL contributes Apo AI, AII, CI, CII, CIII and E to the chylomicron particles. In the presence of Apo CII, LPL becomes activated and breaks down TAGs to glycerol, monoacylglycerol and FFAs. Released FFAs are taken up by muscle cells and adipocytes and used as an energy source or are stored as TAGs, while glycerol is stored for gluconeogenesis, in the liver. TAG metabolism in chylomicron particles decreases the size of these particles and an exchange of Apo AI, AII, CI, CII and CIII into HDL, and leads to the formation of chylomicron remnants (Iqbal and Hussain, 2009).

The endogenous pathway starts in the liver, the central platform for lipid metabolism, whereby nascent VLDL particles are secreted and released into the blood circulation. In the presence of LPL, FFAs and glycerol are released and VLDL is converted into VLDL remnants. The majority of VLDL remnants are returned to the liver via LDLr, while the remainder are converted into IDL particles (Feingold and Grunfeld, 2018). These particles are either reabsorbed by the liver, by LDLr, or hydrolysed to form LDL. As mentioned previously, LDL is the main carrier that circulates cholesterol in the bloodstream. The majority of LDL particles are taken to the liver by LDLr while the remaining LDL molecules are extracted through scavenger pathways. The uptake of LDL by receptors causes the release of free cholesterol, for storage in peripheral cells (Kingsbury and Bondy, 2003).

The reverse cholesterol pathway is an important and protective process in preventing CVD. The main role of this pathway is to transport cholesterol from peripheral cells to the liver, in the presence of HDL. This pathway starts when precursor HDLs are exported from the liver and small intestine into the blood circulation. Precursor HDLs consist of cholesterol, phospholipids, Apo AI and LCAT. In the presence of the scavenger receptor BI (SR-B1), and the ATP-binding cassette transporter GI (ATBCG1), free cholesterol accumulates in peripheral tissues, and HDL particles enlarge in size and perform HDL3. Then LCAT, the enzyme responsible for esterification, converts free cholesterol into cholesterol ester and forms HDL2. Higher HDL2 levels in the circulation are linked with lower rates of developing atherosclerosis. The transportation of HDL2 to the liver happens after that and again SR-B1 receives cholesterol ester and HDL2 becomes HDL3. In the liver, Apo A1 in HDL3 is destroyed by the kidneys and the

product enters the HDL cycle and is called pre- β HDL particles (Kingsbury and Bondy, 2003).

1.3 Hyperlipidaemia

1.3.1 Definition

Hyperlipidaemia refers to abnormal levels of blood lipids that may be caused by either external factors and/or demographic features. Hyperlipidaemia is one of the most frequently stated risk factors for developing CVD (Reiner *et al.*, 2011). The exact relationship between hyperlipidaemia and CVD is unclear, but studies support the hypothesis that high LDLC is a significant factor inducing atherosclerosis development (Libby, 2011). LDLC is an atherogenic lipoprotein that has been used as a biomarker to define the risk of developing CVD. The molecule is a target for the management of dyslipidaemia, as outlined by the European Society of Cardiovascular (ESC) and the European Atherosclerosis Society (EAS) guidelines (Catapano *et al.*, 2016).

1.3.2 Screening and classification of lipid levels

The screening for dyslipidaemia is crucial for individuals with high risks of developing CVD. Any one of the following criteria is classified as high risk: men ≥ 40 years and women ≥ 50 years of age or post-menopausal, especially if other risk factors are present; high blood pressure ≥ 180 systolic /110 diastolic mmHg, diabetes mellitus type 1 or 2 (Catapano *et al.*, 2016). British and American classification of lipoprotein lipid levels are shown below in Table 1.1 (Heart UK, n.d.; Jacobson *et al.*, 2015).

Table 1.1 Classification of lipid levels in mmol/L

	USA classification*	UK classification**
Total cholesterol		
<i>Desirable</i>	< 5.17	≤5
<i>Borderline high</i>	5.17 - 6.18	
<i>High</i>	≥ 6.20	
Non-HDL-C		
<i>Desirable</i>	< 3.36	≤4
<i>Above desirable</i>	3.36 - 4.11	
<i>Borderline high</i>	4.13 - 4.88	
<i>High</i>	4.91- 5.66	
<i>Very high</i>	≥ 5.68	
LDLC		
<i>Desirable</i>	<2.58	≤ 3.0
<i>Above desirable</i>	2.58 – 3.33	
<i>Borderline high</i>	3.36 – 4.11	
<i>High</i>	4.13 - 4.88	
<i>Very high</i>	≥ 4.91	
HDLC		
<i>Low</i>		
<i>Men</i>	< 1.03	< 1.0
<i>Women</i>	< 1.29	< 1.2
TAGs		
<i>Fasting</i>		<1.7
<i>Non-fasting</i>		<2.3
<i>Desirable</i>	< 1.69	
<i>Borderline high</i>	1.69 - 2.24	
<i>High</i>	2.25 – 5.63	
<i>Very high</i>	≥ 5.64	

*National Lipid Association (Jacobson *et al.*, 2015).

**Heart, UK (n.d.)

1.3.3 Measuring lipoproteins in the blood: fasting versus non-fasting

It has previously been accepted that lipid blood samples should be taken during a fasting state. Recently, a study examined whether there were differences between fasting and non-fasting sampling and found that TC and HDL values were similar (Langsted *et al.*, 2008). As blood TAGs are influenced by food, results can be on average 0.2 mmol/L higher, if tested during a non-fasting state (Langsted *et al.*, 2008). Traditionally, LDL is calculated by the Friedwald formula: $LDLC \text{ (mg/dL)} = TC \text{ (mg/dL)} - HDLC \text{ (mg/dL)} - TAG \text{ (mg/dL)}/5$ (Friedwald *et al.*, 1972), which depends on the value of TAGs, therefore any changes in blood TAGs can lead to the underestimation of LDLC

levels. However, this does not apply to the direct measurement of LDLC when a non-fasting measurement will not affect LDLC levels (Mora *et al.*, 2009).

Recent guidelines from the National Institute for Health and Care Excellence (NICE) and ESC/EAS recommended measuring lipid profiles in a non-fasting state when screening for and estimating general CVD risks (NICE, 2014; Catapano *et al.*, 2016). However, for patients with severe dyslipidaemia, especially hypertriglyceridemia, fasting samples are recommended (Catapano *et al.*, 2016).

1.3.4 Causes of hyperlipidaemia

Hyperlipidaemia can be caused by genetic and/or environmental factors. In addition, some diseases and drugs have been reported to elevate LDLC and TAG levels.

1.3.4.1 Genetic factors

Gender

In a study conducted by Duvernoy *et al.* (1999), comprising equal numbers of non-diabetic post-menopausal women (where oestrogen-replacement therapy was being taken by only one woman) and men of matched ages (52 and known cholesterol levels, it was found that women had significantly higher HDL levels ($P < 0.001$), lower TAG levels ($P = 0.01$) and more favourable LDL/HDL ratios ($P = 0.0001$), than men. In addition, Positron emission tomography, an imaging-scanning technique, was employed to detect metabolic body processes. Results showed that women had significantly better myocardial blood flows and higher heart rates, than men. Interestingly, a significant correlation was reported between lipid biomarkers (HDL, TAGs, and TC/HDL ratio) and hyperaemic blood flow (Duvernoy *et al.*, 1999). The mechanism behind this correlation was explained in a previous study. In 1996, Samra and colleagues (1996) investigated the effects of injected epinephrine in six healthy

volunteers (male n=4, female n=2) to investigate interactions between blood flow and lipid metabolism. Epinephrine is a hormone secreted by the adrenal glands that causes increased blood flow throughout the body, including adipose tissues. The study results showed that increasing blood flow to adipose tissues led to a greater delivery of TAG rich lipoprotein particles and hence increased hydrolysis. Moreover, epinephrine causes a significant increase in the rate of LPL functions (Samra *et al.*, 1996).

Ethnicity

Ethnicity has been reported to affect lipoprotein levels in blood. In a South African study conducted by Ellman and colleagues (2015), it was found that HDLC, TAGs, TC, and LDLC were lower in black women when compared to white women. In the study, cholesteryl ester transfer protein (CETP) genotypes were analysed for both groups and results showed that lipid level differentiation between ethnicities was due to CETP gene polymorphisms. The CETP gene polymorphism affects CETP activity which decreases the transfer of cholesterol esters from HDLC particles to LDLC particles, leading to LDLC decreases in blood. A more recent prospective cohort study in multi-ethnicity populations in the US, showed that high levels of Lp(a) were associated with polymorphisms in the LPA gene, in different ethnic groups of Africans and white American people (Lee *et al.*, 2017).

1.3.4.2 Post-menopause

In another cross-sectional study conducted in healthy volunteers, including pre- and post-menopausal women, higher levels of TC, TAGs, non-HDL-C and TC/HDL ratios were reported in the post-menopausal group versus the pre-menopausal group (Anagnostis *et al.*, 2015). These diverse results for pre- and post-menopausal women could be due to lower oestrogen levels after menopause that may impact on liver

lipoprotein transport (Anagnostis *et al.*, 2015). Oestrogen influences lipoprotein metabolism by increasing the performance of hepatic LDLr (Windler *et al.*, 1980). Currently, the NICE recommends that CVD risk assessment should be done every five years after age of 40 even though if there was no history of CVD (NICE, 2014).

1.3.4.3 Environmental factors

In terms of reducing mortality and morbidity connected to hyperlipidaemia, in particular among individuals with low-moderate risks of CVD, 2016 ESC/EAS guidelines (Catapano *et al.*, 2016) for dyslipidaemias management has been suggested that improving one's lifestyle can be used as a therapeutic strategy to control elevated atherogenic lipid levels. Positive lifestyle changes include moderate dietary fat and alcohol intake, weight reduction for overweight and obese people, increasing physical activity and abstinence from smoking (Catapano *et al.*, 2016).

Dietary fat

In relation to CVD, several studies have investigated the relationship between dietary fats and cholesterol levels. Griffin and Lichtenstein (2013) published a review of randomised controlled trials, conducted between 2003 and 2013, and concluded a moderate relationship between dietary cholesterol levels and lipid concentrations in blood plasma including TC, HDLC, TAGs, TC/HDL ratio, but their main focus was plasma LDLC levels. This variation in results was mainly due to differences in study group populations, some studies were conducted among healthy groups while others were carried out among diabetic subjects. However, the SACN (2018) recommends limiting SF and trans-fatty acids intake to 10% and 2% of total energy intake, respectively. Although approximately 15% of cholesterol in the blood comes from dietary sources, it is recommended not to exceed an intake of more than 300 mg cholesterol per day.

Alcohol consumption

The relationship between alcohol consumption and lipidaemia has always been controversial (Buja *et al.*, 2010; Wakabayashi, 2010). A published meta-analysis of the efficacy of alcohol consumption on CVD biomarkers analysed 42 experimental studies conducted among people who were free from heart disease, diabetes or alcohol dependence (Brien *et al.*, 2011). The analysis suggested that the consumption of 30 g of alcohol per day can increase HDL plasma levels by approximately 3.66 mg/dL (0.095 mmol/L), which can reduce CVD risk by 17% (Brien *et al.*, 2011). The role of alcohol in increasing HDLC levels appears to be due to the stimulation of hepatic synthesis and secretion of Apo AI and AII (Branchi *et al.*, 1997). Moreover, heavy consumption of alcohol of more than 60 g per day, can lead to hypertriglyceridemia defined by high levels of circulating TAGs (Brien *et al.*, 2011; Wakabayashi, 2014). These observations can be explained by animal studies which observed that after alcohol ingestion, VLDL particle production increased in hepatic cells in rat models (Daher *et al.*, 2006). Also, chylomicrons decreased in size and changes in cholesterol and phospholipid levels were observed, boosting bile secretion from the liver, causing increased TAG levels (Daher *et al.*, 2006). However, for men and women, recent clinical guidelines have recommended not exceeding 14 units (112 g) of alcohol per week (NHS, 2016).

Body weight and exercise

Being overweight with abdominal adiposity has been linked to hyperlipidaemia (Casanueva *et al.*, 2010). An individual is defined as overweight if their body mass index (BMI) is above 25kg/m² (WHO, n.d.a). A waist circumference measurement is used to assess abdominal adiposity: for men > 94 cm (37 inches) and > 80 cm (31.5 inches) for women, even if the BMI is normal (NHS, 2016). A reduction of 5 to 10% in body weight can address improvement in lipid levels (Dattilo and Kris-Etherton, 1992).

It is always recommended to maintain the body at an ideal weight and a regular physical activity for a minimum of 30 minutes daily (Catapano *et al.*, 2016). Increased weight has been associated with lower HDLC, such that every kilogram of additional weight reduces HDLC by 0.5–1% (Dattilo and Kris-Etherton, 1992; Hession *et al.*, 2009). On the other hand, a one kilogram of weight reduction can increase HDLC by 0.01 mmol/L (0.4 mg/dL; Catapano *et al.*, 2016).

Moreover, increased physical activity is associated with a reduction in CVD risk (Blair *et al.*, 1995). In terms of the effects of regular exercise on dyslipidaemia, a study was performed in 84 dyslipidaemic individuals by Kraus *et al.* (2002). Study participants were assigned to three exercise groups: high volume -high intensity exercise, low volume -high intensity exercise and low volume -moderate intensity exercise for six months. Results showed that TAGs and VLDL levels were decreased for all groups. A significant decrease in LDL particles (4.4%) and significant ($P < 0.017$) increases in HDL cholesterol concentrations were detected in the high volume-high intensity exercise group. This study suggested improvement in lipoprotein profile is related to the volume of exercise more than exercise intensity (Kraus *et al.*, 2002).

Smoking cessation

In a prospective randomised clinical trial, individuals who stopped smoking showed significantly higher HDLC levels ($P < 0.001$) when compared to smokers, despite a significant weight gain in the group stopping smoking ($P < 0.001$; Gepner *et al.*, 2011). This could be attributed to the detrimental effects of smoking on reducing LCAT levels, the enzyme responsible for the cholesterol reverse transport pathway (McCall *et al.*, 1994). The metabolic effect of smoking on HDL metabolism suggested to be behind the smoking effect on increasing the release of the hormone, catecholamine which boosts

the circulation of free fatty acids (FFAs), resulting in higher concentrations of VLDL and LDL particles and lower HDLC concentrations (Campbell *et al.*, 2008). However, some studies show mixed results of the efficacy of smoking on these enzymes (Zaratin *et al.*, 2004).

Drugs and diseases

Some diseases and disorders can lead to hyperlipidaemia and thereby affect concentrations of LDLC and/or TAGs. These diseases include: chronic kidney disease, hypothyroidism, metabolic syndrome, diabetes mellitus, nephrotic syndrome, human immunodeficiency virus infection, obstructive liver disease, autoimmune disorders and polycystic ovary syndrome disease. Pregnancy and low oestrogen level conditions such as the menopause, are also related to hyperlipidaemia. Some pharmaceutical agents (e.g. oral oestrogens, anabolic steroids, retinoids and thiazide diuretics) can raise LDLC and/or TAG levels, as a side effect (Jacobson *et al.*, 2015).

1.3.5 Pharmacological treatment of dyslipidaemia

Many drugs have been used to treat hyperlipidaemia; the most frequently used drugs are statins. Statins work by inhibiting the activity of 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase, which reduces cholesterol synthesis in hepatocytes. Reducing cholesterol concentrations encourages the liver to express LDLr on its surface. As a consequence, LDL in the blood will sequester back to the liver and levels of circulating LDL levels will decrease, along with other lipoproteins containing apolipoprotein B (Apo B) and TAG particles. Statins have been shown to potentially lower the levels of the following molecules: LDLC (37%), TAGs (19%) and non-HDL-C (33%; Jacobson *et al.*, 2015).

Other drugs such as bile acid sequestrants, cholesterol absorption inhibitors and nicotinic acids are also used to treat hyperlipidaemia. These drugs employ different mechanisms to improve lipoprotein levels, which have been shown to be effective in reducing LDL-C, non-HDL-C and TAGs by averages of 18%, 14% and 16%, respectively (Jacobson *et al.*, 2015).

1.4 Phospholipids

Phospholipids (PLs) are classified into two major classes based on their base structure: glycerophospholipids and sphingolipids (Gurr *et al.*, 2016). Glycerophospholipid structures are comprised of a phosphate group (polar head) and two fatty acids (nonpolar tails), with glycerol attached to a group that defines different type of glycerophospholipids, e.g. choline, ethanolamine, inositol, serine, or glycerol. Sphingolipids are the other major class of PLs which have the same structure as glycerophospholipids, but with one sphingosine molecule instead of glycerol (Akoh and Min, 2008). PLs are main component of all mammalian cellular membranes. The most abundant phospholipids found in mammalian cellular membranes are PCs, which account for 40-50% of total PLs (van der Veen *et al.*, 2017). The percentage of PC to total PLs in blood lipid pools is as following: 70%, 35% and 30% of plasma, platelets and erythrocytes respectively (Hodson *et al.*, 2008). PC supplementation has been extensively studied in human health due to its perceived beneficial effects (Küllenberg *et al.*, 2012).

1.5 Phosphatidylcholine

1.5.1 Phosphatidylcholine overview, structure and metabolism

Phosphatidylcholine was discovered by Théodore-Nicolas Gobley in 1847 and is called lecithin, from the Greek word *lekithos*, which means egg yolk (Gobley, 1850). PC is a class of PL and includes, phosphatidyl-ethanolamine (PE), -inositol (PI), -serine (PS) and sphingomyelin. The chemical structure of PC consists of a choline and a phosphate attached to glycerol and two tails of fatty acids (Figure 1.3). PCs vary in their molecular structure, in terms of position and length of double bonds in the fatty acid tail. For instance, in the liver, a typical PC molecule contains a saturated fatty acid in the *sn*-1 position and a PUFA acid in the *sn*-2 position. However, the most common types of fatty acids found in PC molecules (up to 77%) are palmitic 16:0, stearic 18:0, oleic 18:1 and linoleic 18:2 (Phillips and Dodge, 1967). Physiologically, PC is an important component of eukaryotic cell membranes, as it plays key roles in lipoprotein and lipid metabolism (Treede *et al.*, 2007).

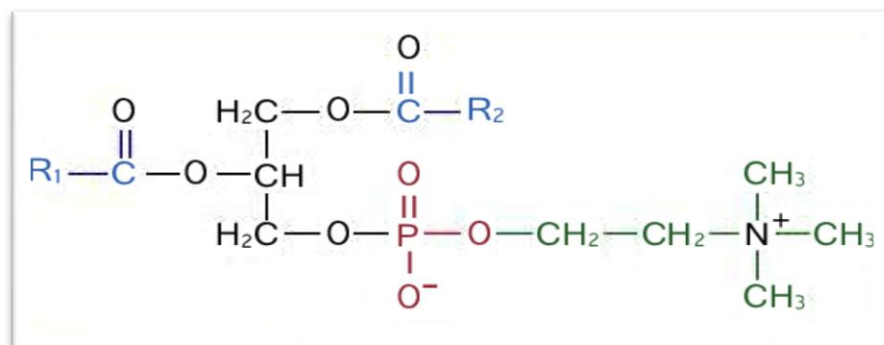


Figure 1.3 The chemical structure of phosphatidylcholine (Siraj *et al.*, 2014)

The most abundant lipid in the intestinal lumen is PC, coming directly after TAG (Phan and Tso, 2001); 90% of exogenous PC is absorbed by the intestine when taken with meals (Zierenberg and Grundy, 1982). Absorption starts when the lumen hydrolyses

the fatty acids in phospholipids via pancreatic phospholipase A2 enzymes from the second carbon group of glycerol. As a result of hydrolysis, FFAs and lysophospholipids (lysoPLs) are formed (Richmond *et al.*, 2001). Then, mucosal cells absorb FFAs and lysoPLs and enter enterocytes, where both are re-esterified to form PLs that enter the bloodstream (Cohn *et al.*, 2010). There, PLs integrate with chylomicrons, while small proportions integrate with VLDLs. However, 20% of intestinal PLs can be absorbed directly, without hydrolysis, and incorporated into HDL. In the liver, HDL is hydrolysed in the presence of LCAT and re-esterified to form PLs to be carried to surrounding tissue as VLDL. In addition to the transport of PL absorption molecules, the cellular membrane, PLs, in particular PC, can be secreted in bile. Biliary PLs are crucial for the emulsification and absorption of lipid soluble molecules, such as cholesterol.

The exact metabolism of PLs, and the mechanism of their incorporation into cellular membranes has not been well characterised. There is a requirement to define the metabolic pathway of each PL type, but this is difficult, as PLs are naturally found in a wide range of foods items and also include lipid components, especially TAGs.

1.5.2 Phosphatidylcholine sources

1.5.2.1 Synthesis

In humans, PC can be biologically synthesised in two ways: the main pathway is via the Kennedy phosphatidylcholine pathway, also known as the CDP-choline pathway (Kennedy and Weiss, 1956).

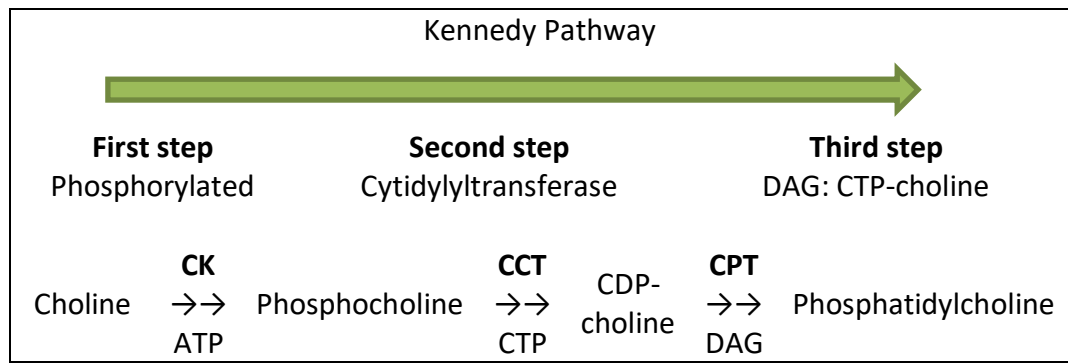


Figure 1.4 Kennedy (CDP-choline) pathway

This pathway starts when exogenous choline sources enter mammalian cells (Figure 1.4) via choline kinase (CK). Choline is converted into phosphocholine in the presence of adenosine triphosphate (ATP). Then, the rate-limiting enzyme CTP:phosphocholine cytidylyltransferase (CCT) mutates choline phosphate to produce CDP-choline. In the final step, cholinephosphotransferase (CPT) forms PC using CDP-choline and DAG (Gibellini and Smith, 2010).

PC can also be synthesised by another minor pathway in hepatic cells, the PEMT pathway. In this pathway, PC is synthesised via three sequential methylations of PE catalysed by PE-N-methyltransferase (PEMT; Pynn *et al.*, 2011). Although hepatocytes are the main cell responsible for PEMT activity, small levels of PEMT activity have been recorded in adipocytes (Cole *et al.*, 2012). Approximately 70% of endogenous PC synthesised in the liver is produced via the CDP-choline pathway, while 30% comes from the PEMT pathway (EFSA, 2016). Previous studies have demonstrated that when the Kennedy pathway is functional, the PE methylation pathway is not required for hepatic lipoprotein secretion. This means that the PEMT pathway can act as a substitution if the CDP-choline pathway is blocked (Yao and Vance, 1988).

The synthesis of PC is important to human health. Modulation of PC synthesis is associated directly with membrane and cellular dysfunction (Fagone and Jackowski,

2013), along with serious diseases such as alcoholic fatty liver (Lieber, 2004) or non-alcoholic fatty liver (Song *et al.*, 2005). In a recent case study (Payne *et al.*, 2014), two unrelated female cases presented with severe non-alcoholic fatty liver disease, lipodystrophy, very low levels of HDL, short stature and insulin resistant diabetes. Investigations included the measurement of PC synthesis along the Kennedy pathway in fibroblasts. The results showed a significant reduction in PC synthesis (Payne *et al.*, 2014). Moreover, a reduction in PC synthesis could lead to apoptosis, especially in neuron cells (Cui *et al.*, 1996). Previous studies have described low plasma PC levels in serum from Alzheimer's disease patients (Whiley *et al.*, 2014) and Huntington's disease patients (Cheng *et al.*, 2016).

1.5.2.2 Dietary sources

To date, the only food composition database for PC is available from the Agricultural Research Service at the US Department of Agriculture (USDA; Patterson *et al.*, 2008). PC can be found in many foods, but the major dietary source of PC is egg yolk, as 100 g can contain as much as 630 mg PC. It is also found in higher concentrations in whole eggs and beef liver at 250 mg/100 g. Small amounts of PC are also present in fruit and vegetables (Table 1.2).

Table 1.2 Top ten food sources of PC in animal and vegetable products (mg/100g)

Ranking	Animal products		Vegetal products	
	Food	Content (mg/100g)	Food	Content (mg/100g)
1	Egg, yolk	630	Mustard seeds	75
2	Liver, Beef	250	Parsley	72
3	Eggs, whole	250	Soybean	65
4	Liver, chicken	210	Pistachio	51
5	Bacon, pork	89	Edamame	46
6	Shrimp	67	Pine nut	45
7	Loin, Pork	62	Almond	40
8	Steak, beef	60	Cashew	37
9	Fish, tilapia	54	Flaxseed	36
10	Fish, salmon	48	Peas	24

Source: USDA database, 2008

Apart from natural foods, PC can be found in processed food under the name of lecithin. Lecithin is a food additive and is used as an emulsifier in the food industry (Whitehurst, 2004). Lecithin has been regulated by the European Union, under regulation 1333/2008 and is known as E322. This additive is defined as a mixture of PLs from either animal or vegetable food sources. It is derived by physical processes additionally to hydrolysed substances that are obtained by the use of safe and suitable enzymes. The final product of lecithin should not have any residual enzyme activity (EFSA, 2017). Lecithin can be found in processed foods such as margarine, pastries, sauces, cheese, infant formula and also found in chocolate. Lecithin can be used at 1% of the final food preparation (Leray, 2014).

1.5.2.3 Supplementation

PC supplements in the form of capsules, granules and liquids can be manufactured using natural food such as soybeans, sunflower seeds, rapeseed and hens eggs (Leray, 2014). The most common supplement is commercially known as lecithin, which is usually made from soybean powder that has been processed and modified to reduce the risk of allergy linked to soybean, as well as removing genetically modified products. Lecithin contains up to 80% PC (Leray, 2014). These supplements are approved by the U.S Food and Drug Administration (FDA, 2016). These supplements can be beneficial for individuals who are trying to reduce their dietary cholesterol intake by limiting the consumption of eggs and meat, the two main dietary sources of PC.

1.5.3 Safety and toxicity

Lecithin is recognised as being safe and is approved by the U.S Food and Drug Administration (FDA, 2016). Many animal and clinical trials have been conducted and have indicated that PC is non-toxic and is well tolerated even when used in very high

doses, over a long time. In a previous study, 48 g of PC was consumed for more than 24 months with no significant side-effects reported (Tompkins and Parkin, 1980). Another review suggested that 18 g of PC per day is well tolerated, whereas PC intolerance symptoms may manifest as diarrhoea, nausea, and bloating (Kidd, 2002).

1.5.4 Recommended daily dietary phosphatidylcholine intake

There is currently no recommended and reference intake data for PC in the UK. This may be due to the lack of reliable studies on PC consumption and absorption. In 2016, the EFSA panel considered setting Dietary Reference Values (DRVs) for choline, where PC is the main component. However, data uncertainty regarding human need for choline or a dose-response relationship between exogenous intake and plasma status concentrations were barriers to setting DRVs for choline. However, the panel decided an adequate intake (AI) was 400 mg/day (EFSA, 2016). The adequate daily intake for adults in the US is estimated to be 550 mg for choline, which is equivalent to 4 g of PC (Leray, 2014). Therefore, it is suggested that for the European population, the AI for PC is approximately 3 g.

1.5.5 Phosphatidylcholine functions

The systematic importance of PC in the human body lies in two areas. First, it is a main component of the lipid bilayer in all cell membranes, as it is located in the external layer of cellular membranes, where it is critical for structural integrity and function. Second, PC plays a vital role in VLDL synthesis via hepatic secretion, with an essential role in the distribution of cholesterol and other lipids to body tissues. Additionally, this phospholipid is one of the biliary salts that help the absorption of lipo-soluble molecules (Castro-Gómez *et al.*, 2015).

The first evidence that reduced PC synthesis impaired the hepatic secretion of lipoproteins was observed when rat hepatocytes were cultured, post a three day choline-deficient diet (Yao and Vance, 1988). Study results showed that hepatic PCs were lower by 25% and TAGs had accumulated in the liver by up to 650% when compared to control rats. Later, isolated hepatocytes were supplemented with choline and a rapid correction of PC synthesis, via CDP-choline pathways, was reported along with a resumption in VLDL secretion. This observation suggested that the accumulation of TAGs was due to the inhibition of hepatic VLDL secretion (Yao and Vance, 1988). Subsequent studies have confirmed that the significant impairment of hepatic VLDL secretion (~50% less Apo B100), that causes hepatic TAG accumulation and reduced plasma TAG levels is due to impaired PC biosynthesis from either CDP-choline or PEMT pathways (Waite *et al.*, 2002; Noga and Vance, 2003a; Jacobs *et al.*, 2010).

1.5.6 Phosphatidylcholine health effects

Many studies have been conducted focusing on the health benefits of PC, either *in vitro* or *in vivo* (Castro-Gómez *et al.*, 2015). The beneficial effects of PCs have been examined in cancer, liver disease, CVD and lipid management. Additionally, PCs have been explored in terms of improving brain and cognitive behaviour, as well as improving some aspects of Alzheimer disease.

1.5.6.1 Brain development and cognition

During ageing, the lipid composition of brain cells changes (Svennerholm *et al.*, 1997). Choline, a main component of PC, is vital for brain tissue development. During an *in utero* study, reductions in circulating choline concentrations were observed during brain development, indeed choline concentrations in plasma and serum were higher in neonates and foetuses when compared to adults (Zeisel, 2004).

In an interventional study by Ladd *et al.* (1993), a 25 g of PC supplementation was administered to 80 college students, followed by series learning tasks to measure their cognitive abilities. A significant improvement in memory was observed at 90 minutes post ingestion. This study suggested that PC supplementation could help slow learners and patients with neurological disorders. In a study by Nagata *et al.* (2011), the effects of a daily dose of 50 mg/kg of PC were examined in 310 subjects aged between 59 and 95 years, with age-related cognitive disorders. After five months, a mini-mental state examination suggested that PCs could improve spatial learning and memory impairment and dementia in humans.

Memory and cognition impairment are commonly observed in Parkinson's patients (Kehagia *et al.*, 2010). In one study, a soybean lecithin granule (containing 25% PC) was administered to sixteen patients with Parkinson's disease, over a nine week period. Although there was a negative improvement regarding the progression of the disease itself, there was a positive effect regarding memory, cognition and motility tests (Tweedy and Garcia, 1982).

1.5.6.2 Cancer

Li and colleagues (2006) found that cancer cell membranes acquire particular features that differentiate them from those found in progenitor cells. This enables malignant cells to separate from surrounding tumour tissue and migrate to other tissues; a process called tumour metastases. Membranes from cancer cells have higher concentrations of cholesterol when compared to other non-cancerous cells; this was related to higher apoptotic sensitivity (regulated by cholesterol content). Subsequently, the control of the composition and density of lipid rafts could

potentially change the viability of the malignancy cells and modify metastatic behaviour (Li *et al.*, 2006).

In an *in vitro* study, Sakakima *et al.* (2007) treated human hepatocarcinoma cells for growth inhibition in the presence of soybean PC (95% pure PC) or egg yolk PC (99% pure PC) with menaquinone-4 (vitamin K2). In the same study, treatment of PC and menaquinone-4 was tested in rat and showed PC administration alone can significantly have an effect on growth inhibition. However, combining PC with menaquinone-4 has a greater significant effect (Sakakima *et al.*, 2007). These results point to the potential of using PC supplementation for hepatic cancer patients' in future clinical trials.

1.5.6.3 Non-alcoholic fatty liver disease

Approximately 20-30% of UK population have been diagnosed with non-alcoholic fatty liver disease (NAFLD; NICE, 2016), even with little or no alcohol consumption. NAFLD is a medical condition defined by the accumulation of TAGs in more than 5% of hepatic cells (Green *et al.*, 2018). Until now, therapeutic options for NAFLD were limited to hypocaloric diets and high physical activities and hyperglycaemia control (Gitto *et al.*, 2018). However, PCs have been widely explored as effective treatments of NAFLD, by targeting fat accumulation in the liver (Gundermann *et al.*, 2011). In 2008, a randomized controlled trial was performed among 40 patients with mixed FLD diagnoses; 25 patients were administered 1.7 g metformin plus 600 mg PC per day and 15 patients were administered metformin only (Buyeverov *et al.*, 2008). Metformin is a hypoglycaemic agent that has been used for treating NAFLD because of its efficacy in improving the metabolic and biochemical features among NAFLD patients although it does not improve the histological status of the liver (Li *et al.*, 2013). After six months, liver enzymes in the PC group were significantly lower compared to the control group

($P < 0.05$; Buyeverov *et al.*, 2008). In the same year, Sun and colleagues performed a randomised controlled trial for three month among 74 diabetic patients with NAFLD. Half of the patients ($n=37$) were treated with 600 mg PC plus 500 mg metformin and the other group ($n=37$) received metformin only. At the end of the treatment phase, both groups had significant improvements in term of TCs, TAGs and liver appearance (ultrasound); however, in comparison between the two groups the PC group shown a significant improvement in hepatic lipid (TC and TAGs $P < 0.01$) comparing with the metformin only group (Sun *et al.*, 2008).

The positive and antioxidative effects of PC on NAFLD are believed to be caused by the renormalisation of PC/PE ratios in hepatic cellular membranes. Abnormal distribution of PC/PE ratios may be associated with the inflammation of fatty liver (steatohepatitis), through adversely affecting plasma membrane permeability, which exposes the hepatocellular content to the cellular space (Li *et al.*, 2006).

The main sources of PC used in interventional studies are egg PC and soybean PC. The effectiveness of these two PC sources was studied in animal study (Tandy *et al.*, 2010). where twenty five mice were divided into five groups (5 mice per group): i) high fat diet alone or ii) high fat diet supplemented with either egg PC, iii) soybean PC, iv) hydrogenated egg PC or v) hydrogenated soybean PC. The PUFA content for each group was: 70%, 20% and 0% for soy, egg and hydrogenated PC groups, respectively. After three weeks of supplementation period, liver samples was analysed and the results showed that the total hepatic lipids were significantly lower in hydrogenated soy and egg PC groups, when compared to non-hydrogenated groups ($P < 0.05$). Similarly, TC and TAG hepatic levels were lower in hydrogenated group. These results suggest that the beneficial effects of PCs in lowering liver lipids are not due to the

PUFA content. Therefore, there are no systematic differences between PCs derived from soy or egg yolk (Tandy *et al.*, 2010).

1.5.6.4 Cardiovascular and lipid management

The concept of PC supplementation, either as lecithin or pure PC, to treat abnormal lipid metabolism and CVD risk has been studied since 1956 (Davies and Murdoch, 1956). Since then, oral PC supplementation has been extensively explored in the literature and a significant reduction effect on atherogenic lipoprotein and rise in HDLC levels have been reported. In an early study by Childs *et al.* (1981), it was reported that 10.4 g of PC, over two months, significantly increased HDLC levels in six hypercholesterolemia patients ($P < 0.02$), while a simultaneous decreasing trend in LDLC levels was observed. In addition, Medić *et al.* (2003) examined the effects of daily doses of the soybean 'Leci-Vita' product (1.2 g PC) on 106 dyslipidaemic patients. After 24 weeks, a significant ($P < 0.001$) improvement in TC and HDLC levels were recorded. In another study by Høie *et al.* (2005), the effects of administering 2 g PC to 39 hyperlipidaemic adults was investigated. At the end of the eight week trial, a significant decrease in LDLC ($P = 0.001$) and TC ($P < 0.001$) was recorded when compared to the placebo group ($n = 38$). Another study by Spilburg *et al.* (2003) reported significant reductions in TC ($P = 0.001$) and LDLC levels ($P = 0.001$) when 1.9 g soy PC was administered to 24 subjects, with high levels of LDLC over four weeks.

In contrast, other studies have not observed such effects in lowering atherogenic lipoproteins. For example, in 1998 a double-blind study was conducted among 21 hyperlipidaemic men (Oosthuizen *et al.*, 1998). Participants were assigned randomly to one of three groups: i) frozen yogurt; ii) frozen yogurt with 17 g sunflower oil; or iii) frozen yogurt with 20 g of soybean lecithin containing 96% PLs. After four weeks of

treatment, soybean lecithin had shown no effects on reducing serum atherogenic lipoproteins (TC, TAGs, and LDLC). However, this result might be limited to the small number of participants in each group as well as to the amount of PC in the soybean lecithin.

Recently, corroborative evidence starts to appear on the role of dietary PC metabolism in developing CVD by producing trimethylamine N-oxide (TMAO) gas. A prospective clinical study conducted by Tang *et al.* (2013) investigated the relationship between dietary PC intake, TMAO level and CVD risk in humans. The study was conducted among 40 healthy participants who completed three visits of a dietary PC intervention, which consisted of two large boiled eggs along with 250 mg of deuterium-labeled PC (d9-PC), as a tracer, provided in a gelatine capsule (Tang *et al.*, 2013). In each visit venous blood sampling was collected at 1, 2, 3, 4, 6, and 8 hrs after baseline to measure fasting TMAO levels (Tang *et al.*, 2013). The results indicated higher levels of plasma TMAO after the dietary PC intervention due to the formation of the metabolite trimethylamine (converted later to TMAO) by intestinal microbiota during the dietary PC metabolism (Wang *et al.*, 2011). Furthermore, a clinical study (Tang *et al.*, 2013) was carried among 4007 adults for three years follow up to explore the correlation between fasting plasma TMAO levels and the incidence of a major adverse cardiovascular event (for example, stroke, myocardial infarction, or death). Results showed a strong correlation ($P < 0.001$) between high levels of TMAO and increased risk of a major adverse CVD event. After adjusting for CVD traditional risk factors, elevated levels of TMAO remained significant independent risk factors (Tang *et al.*, 2013). The exact mechanism behind the role of TMAO in CVD and atherosclerosis in particular is limited. However, it is suggested that TMAO facilitates foam cell formation

within the arterial wall. Additionally, it was reported that TMAO also inhibits cholesterol efflux by restraining reverse cholesterol pathway, as well as by blocking bile acid secretion (Liu *et al.*, 2015).

A more recent study found that dietary PC had no effect on TMAO levels (Lemos *et al.*, 2018). A cross-over study was conducted among thirty healthy participants, to explore the effect of egg consumption (3 eggs per day) or choline supplementation on TMAO concentration for four weeks. Serum levels of TMAO were measured at baseline and end point of each intervention phase. The results showed that egg consumption and choline supplementation have no effect on fasting TMAO levels ($P = 0.874$).

The relationship between dietary PC and TMAO formation can be affected by several factors that influence the concentration of TMAO levels in the blood. These factors include dietary habits, gut microbiota, liver flavin monooxygenases activity, and kidney function (Canyelles *et al.*, 2018). However, further work to understand the natural variation of TMAO metabolism in different populations is needed.

1.6 Food frequency questionnaire

Studies that have investigated the effects of PCs on human health have mostly been interventional studies (Olthof *et al.*, 2005). There is limited literature assessing the relationship between dietary PC intake with disease mortality or morbidity. There is a need to establish a valid tool to measure dietary PC in human diets, and a requirement to establish associations between dietary PC intake and disease development.

1.6.1 Definition

The food frequency questionnaire (FFQ) is a common dietary assessment tool used in research since the 1990s (Shim *et al.*, 2014). The aim of any FFQ is to estimate the

consumption frequency of specific food items listed on the questionnaire. This method is commonly used in epidemiological studies to investigate relationships between specific nutrients and disease development. To perform a reliable dietary assessment and avoid false ideas about dietary factors and disease development, FFQs should be robustly developed, designed well and validated by peer review (Willet, 2012).

1.6.2 FFQ components

1.6.2.1 Food list

To devise a successful questionnaire, it is crucial to choose the correct food list. Before choosing such a list, it is important to consider the target population and the questionnaire's aims. Based on these factors, a wide variety of nutrients and food groups of interest should be included in the FFQ. In previous questionnaires, food lists have varied widely, where food items have ranged between five and 350, with an average of 88 food items (Cade *et al.*, 2004). Listing food items in a questionnaire should be performed carefully to prevent respondent confusion and to ensure questionnaire results are accurate. This means that related foods should be merged together, such as dairy products or grains. For food items that are closely related, specific items should be placed before general items (e.g. low-fat cream before other creams).

1.6.2.2 Frequency in a questionnaire

Assessing frequency using a questionnaire is helpful for respondents to remember the frequency of food item consumption. Frequencies must indicate the intake over the period in question. It is important to keep frequency categories sequential with no gaps; if a respondent finds a gap, this may lead to confusion and inaccurate results. Frequency choices usually range between one and twelve sections, but a commonly

used frequency is nine categories (Cade *et al.*, 2004). An example of nine categories is six or more per day, four to five per day, two to three per day, one per week, one to three per month, less than once a month or never. Some food items may need to have a separate part that asks about consumption and for more explanation from the respondent to collect proper results. For example, seasonal foods (e.g. watermelon) can be eaten daily when in season, but never out of season. Also, milk may need more explanations when it is, for example, used in beverages or on cereals.

1.6.2.3 Portion size

An FFQ can be either qualitative or quantitative. Qualitative FFQs do not include portion sizes, whereas quantitative FFQs do (Pérez Rodrigo *et al.*, 2015). Portion sizes are measured by household units (e.g. cups), food models or other such pictures. Semi-quantitative FFQs include portion sizes only for specific foods items or beverages, to assess the usual intake of specific items (Pérez Rodrigo *et al.*, 2015). According to the literature, 42% of FFQs specify portion size, 36% of questionnaires leave portion sizes open, meaning these FFQs allow participants to describe their portion sizes and the remaining 22% are qualitative (Cade *et al.*, 2002).

1.6.3 FFQ administration

FFQ administration can be performed either by interviewers (face-to-face or telephone) or self-administered, according to study requirements. Web-based forms are also an option for FFQs, these can have the advantage of reducing the number of missing answers as well as reducing data entry times. The only issue with self-administered FFQs is the high rate of missing answers, leading to blank pages. This issue is solved with an immediate checkout for a questionnaire once it is returned, so it

can be sent back, in the case of missing data. In a previous review, 67% of validation studies used a self-administered method (Cade *et al.*, 2002).

1.6.4 Validity

FFQs should be evaluated and assessed for validity before being used in epidemiological studies as a dietary assessment tool. Validation studies should be performed in participants that represent the same target population as the main study. For validation, FFQs should be measured in reference to other methods.

In a systematic review of 227 FFQ validation studies, approximately 71% were validated against another dietary assessment method. In 19% of these studies, FFQs were validated against biomarkers (blood, urine or both) while other studies (12%) were validated against either energy expenditure or doubly-labelled water. One study used adipose tissue as a reference method against FFQs, measuring the intake of fat-soluble vitamins or fatty acids (Cade *et al.*, 2004). Among dietary assessment tools, weighed food records (WR) are a gold standard (Willet, 2012). The average days used in recording WR was between two and 28 days (Cade *et al.*, 2004).

To determine the validity of an FFQ, a comparison between the FFQ and a reference method should be made. Although 83% of 227 validation studies used correlation coefficients to measure the strength of an FFQ, the Bland-Altman method has been recommended as an indicator of FFQ validity (Cade *et al.*, 2002). Whilst valid, correlations only measure the strength of the relationship between an FFQ and the reference method, whereas the Bland-Altman method measures the agreement between the two tools (Cade *et al.*, 2002). The Bland-Altman test calculates the mean differences of all subjects between the FFQ and the reference method using the formula (Flood *et al.*, 2004):

$$\text{mean (FFQ – reference method)} / \text{mean [(FFQ + reference method)/2]}$$

A significant (P value ≥ 0.05) agreement between two methods indicates a normal sample distribution (Hanneman, 2008).

The sample size for validation studies should be carefully calculated but intuitively, a higher number of participants will generate a better validity value. In a review, validation study sample sizes varied from six to 3,750 participants, with a median of 110 subjects. However, it is recommended that sample sizes for such studies should be conducted with at least 50-100 subjects (Cade *et al.*, 2002).

1.6.5 Advantages and limitations of FFQs

Like any other dietary assessment tool, FFQs have their strengths and limitations. FFQs are cost-effective, especially for large-size studies as they can be self-administered. FFQs are time-saving, as the average time taken to complete a questionnaire is usually 20–30 minutes (Cade *et al.*, 2004). In addition to their strengths, FFQs do not influence respondent dietary behaviours, as they measure nutrient intake in the past. Moreover, FFQs are flexible, they are applicable to paper- or computer-based formats and for data analysis, FFQs are easy to code if they are closed-ended, as it is easier for non-nutritional experts to enter data (Willet, 2012).

The main limitation of FFQs is that they are memory dependent. The tool relies on respondents to remember and describe their diets over a long period, which can be challenging for children and the elderly (Willet, 2012). For researchers, developing and designing FFQs is effort and time consuming. Therefore, the process requires accurate evaluation before administration. Moreover, an FFQ's validity for one population does not mean it can be used on another population, e.g. different dietary habits may be at

play in a new group. Moreover, within the same population, there can be cultural and ethnicity differences leading to different dietary habits (Cade *et al.*, 2002).

1.7 Rationale and aims of the study

This study is important as it investigates the relationship between dietary PCs and lipid profiles in the UK population. The prevalence of hyperlipidaemia is increasing globally and the economic cost of the governments is increasing in term to reduce the risk of CVD. Although hypolipidaemic drugs are available, they do not fully achieve the required goal of LDLC reduction. Also, they cause adverse effects such as increasing the risk for diabetes mellitus (Sattar *et al.*, 2014). Diet and nutrition play vital roles in hyperlipidaemia, with many studies exploring alternative ways to treat high levels of lipidaemia (Huang *et al.*, 2011). Previous human and animal studies have shown that PC supplementation has significant beneficial effects on improving atherogenic lipoprotein and increasing HDLC levels (Spilburg *et al.*, 2003; Høie *et al.*, 2005). Additionally, some studies conducted in the US have looked into the relationship between dietary PC and homocysteine (tHcy) levels; an independent risk factor for CVD (Dalmeijer *et al.*, 2008).

PC can be obtained over the counter in different forms: capsules, granules or liquids. However, the nutritional aspect of PC is very limited, there have been no valid tools to measure PCs from food. Therefore, to the best of our knowledge, this study is the first to construct a valid FFQ to measure PC dietary intake in the UK and it is the first study to estimate the average dietary intake of PCs. Also, this is the first study in the UK to explore relationships between dietary PC intake and cardio-related biomarkers in healthy individuals. In addition, the intervention study is the first of its kind to examine two different doses of PC for mild hyperlipidaemic individuals.

1.7.1 Study objectives

The main aim of this project was to investigate the relationship between PC intake from either diet or lipid supplements, including TC, LDL, HDLC, non-HDL-C and TAGs, among hyperlipidaemic and healthy individuals.

The secondary aims were:

- To validate a self-administered FFQ that may help set PC recommendations for the UK population.
- To estimate the average daily intake of dietary PC among a sample of the UK population.
- To investigate dietary PC as assessed by FFQ in relation to lipid and cardiac-related markers (including LDLC, HDLC, total cholesterol, TAGs, and Lp(a)) in a healthy UK population.
- To investigate the relationship between PC intake from the diet and PC concentrations in the blood.
- To investigate the effects of using two PC doses (600 mg and 2,400 mg) as a preventative supplement for individuals with mild elevated LDLC.
- To determine the washout period of PC effects on lipid profiles after four weeks of supplementation.

Chapter 2: Validation of a self-administrated food-frequency questionnaire to assess dietary intake of phosphatidylcholine (Study 1)

2.1 Introduction

Phosphatidylcholine (PC) is an important component of the cellular membrane (Küllenberg *et al.*, 2012). It can be synthesised in the body (Payne *et al.*, 2014) and is found in a variety of foods, e.g. plants (e.g. soy bean, broccoli and oat bran) and animals (egg yolk, chicken liver and beef liver; Zeisel *et al.*, 2003). Previous research, conducted in the US, has established a relationship between the dietary intake of PC and cardiovascular mortality (Zheng *et al.*, 2016) and tHcy a CVD risk factor (Cho *et al.*, 2006; Chiuve *et al.*, 2007). These studies assessed dietary PC by FFQs which assessed habitual food intake rather than measuring dietary PC (Rimm *et al.*, 1992). Currently, there is a gap in the literature relating to PCs, especially in the area of recommended daily intake, and the provision of a database that provides food PC concentrations in the UK.

FFQs are often used in nutritional epidemiological studies that focus on diet and disease (Willet, 2012). FFQs are low in cost and easy to administrate. Usually FFQs can be created, adapted from previous studies, or modified to match the study population (Cade *et al.*, 2002). Validation studies are important in measuring questionnaire errors and deciding how well the instrument measures what it is designed to measure. To assess the true validity of an FFQ, it must be compared with a reference method (Cade *et al.*, 2002).

To determine relationships between dietary intakes of PC and the risk of developing CVD, a valid nutritional tool measuring the dietary intake of PC is required. In this study, the aim was to validate a FFQ to measure the daily dietary intake of PC. To date, the PC content in food is not recorded in the McCance and Widdowson's database of nutrient content in UK foods (McCance and Widdowson's, 2002). The only available food composition database for PC is from the Agricultural Research Service at the U.S. Department of Agriculture (USDA), issued in January 2008 (Patterson *et al.*, 2008). Considering that FFQ should be valid against reference method (WR) and the PC content of foods is not available in the nutrition analysis software (Nutritics) where the WR would be analysed, therefore it was crucial to include another nutrient to the FFQ, for validation purposes.

Saturated fat (SF) was chosen to be combined with PC as it has shown a strong correlation in a previous study (Feunekes *et al.*, 1993). In this study, the dietary intake of PC was assessed by a newly modified FFQ whose design was adapted from a previous validated questionnaire (Salvini *et al.*, 1989). Modifications were made to meet the UK population food intake. Since WR is considered the gold standard in FFQ validation studies, this was chosen as a reference method (Willett, 2012).

This study is important as it was the first study to develop and validate a self-administered FFQ to measure dietary PC intake. A validated FFQ is a major factor in addressing the relationship between dietary PC intake and morbidity and mortality from heart and CVDs.

In this study, a new FFQ was designed to assess the daily intake of PC by correlating the dietary intake of SF with the intake from WR. Secondly, the aim was to measure the intake of dietary PC among the UK population, using the validated FFQ.

2.2 Methodology

2.2.1 Study participants and design

The validation study was conducted in 104 healthy adults between the ages of 18 and 65 years. Participants were recruited between 1st February 2017 and 10th November 2017. A study advertisement was posted in different places throughout Oxford Brookes University (OBU) and public places around OBU, such as supermarkets and cafés. Similarly, the study was announced in the OBU Research Activity Group and through a local Oxford city website (<http://www.dailyinfo.co.uk/>).

The exclusion criteria were chronic conditions (diabetes, hypertension, renal disease, liver disease and heart disease) and pregnancy. Ethical approval for the study was obtained from the University Research Ethics Committee (UREC) at OBU (Appendix 1a).

Participants were required to come for two visits to Oxford Brookes Centre for Nutrition and Health (OxBCNH) at OBU. At the first visit, they were asked to sign a consent form (Appendix 1a) and complete a standard health questionnaire (Appendix 4a). Next, they were provided with a food weighing scale and food diary (Appendix 7) to complete a weighed food record for five consecutive days. At the second visit, participants were asked to complete a self-administrated FFQ to measure dietary PC intake (Appendix 8). Participants completed the study within 10 days of starting the study.

2.2.2 Development and administration of the food frequency questionnaire: (tested method)

The FFQ for this study was an adjusted version of a previously validated questionnaire (Salvini *et al.*, 1989). Modifications were performed to meet the aims of the study and to match the foods consumed in the UK population, focusing on foods containing high levels of PC and SF. These modifications included excluding foods with no PC or SF content (e.g. wine and coffee), adding food items high in PC and SF (e.g. amaranth and coconut milk) and adding commonly consumed foods in the UK population (e.g. hash brown and baked potato). PC and SF food contents were taken from the US Department of Agriculture's nutrient database (USDA). This resource provided a list of foods containing PC (released 2nd January 2008) and SF (released 28th October 2016). The database was chosen as it showed PC levels in common foods. A total of 136 food items were included in the FFQ.

The FFQ (Appendix 8) was created to assess intake of PC and SF over the previous year. The list of foods covered sixteen groups: dairy and egg, cereals and grains, fruits and vegetables, legumes, meats and fish, soups and sauces, fat and nuts, spices and herbs, baked products, fast food and finally sugar and sweets (Table 2.1). Portion size options were provided using household measurements (e.g. cup, slice, teaspoon) or by size (small, medium, large). These measurements were explained to participants by using plates, spoons, household measurements or photographs. Questionnaire frequencies were categorised into nine options, ranging from 'almost never' up to 'more than six times per day'. In the beginning, each participant had the FFQ explained to them and the correct way to complete it. Food items were represented by pictures, for those participants for whom English was not their first language. This FFQ was self-

administered and took approximately 20–30 minutes to complete. The researcher checked the FFQ with the participant, for any missing items or pages.

All FFQ responses were entered into a Microsoft Excel spreadsheet and the VLOOKUP formula was used to calculate the PC and SF content for each food item, depending on the frequency of consumption (Miller *et al.*, 2010). Scores were calculated by multiplying the relative frequency for each food item by the nutrient content of the portion sizes. Those items that were left blank in the FFQ were left blank in the spreadsheet.

Table 2.1 Food items assessed in the food frequency questionnaire

Dairy and egg Milk, whole Milk, 2% fat Milk, skim Coconut milk Soy milk Yogurt, plain Yogurt, fruit Yogurt, fruit, non-fat Cottage cheese Cheddar cheese Cream cheese Sour cream Butter with salt Eggs, whole Egg, yolk Cereals/grains Amaranth Barley malt flour Brown rice Egg noodles Oat bran raw Plain oats Quinoa cooked Wheat germ White Rice, cooked Fruits Apples Apricot Avocados Bananas Blueberries Cantaloupe Grapefruit Kiwi Oranges Peaches Pears Prunes Raspberries	Prunes Raspberries Pears Prunes Raspberries Strawberries Mandarins Orange juice Apple juice Vegetables Edamame Artichokes Asparagus Beets, canned Broccoli Brussels sprouts Cabbage Carrots Cauliflower Baked potatoes Mushrooms Onions, sautéed Peas, frozen Green beans Spinach Sweet pepper Sweet potatoes Yellow corn Zucchini Legumes Beans, kidney Beans, navy Soybean, raw tofu, soft Meat Chicken hot dogs Chicken liver Chicken(no skin) Chicken (w/skin) Liver, pan fried Steak Minced beef	Pork loin Pork sausage Bacon Fish Shrimp Finfish Salmon Fish stick Tuna Soup Chicken noodle soup Hot and sour soup chowder soup Sauce/fat Ketchup Tomato sauce Tomato paste Mayo, low fat Mayo, regular Peanut butter Soy oil Fast food Cheese pizza Chicken nuggets hamburger Tacos Lasagne with meat Hotdog and bun Nachos with cheese Potato chips Mashed potato Hash browns	Spices and herbs Chilli powder Curry powder Mustard seeds Parsley dried Turmeric Sweets/Snacks Ice cream Vanilla shake Frozen yogurt Milk chocolate Snickers Corn chips Crisps Popcorn Pretzel, salted Granola Bar Nuts and seed Almond Cashew Flax seed Peanuts Pecans Pine nut Pistachio Walnut Baked products Wheat bread White bread Chocochip cookie Tortillas English muffins Wheat cracker Plain cracker Danish pastry Apple pie Chocolate cake, prepared Pancakes plain Frosted cake Plain muffins Blueberry muffins
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2.2.3 The 5-day weighed food record: (reference method)

Each participant was instructed on the right way to weigh food. A digital kitchen scale accurate to 1g/1ml (Argos Ltd. Milton Keynes, UK) was given to each participant along with a blank sheet to record a 5-day food intake (Appendix 7). Written and verbal instructions on the correct way to record weighing's were provided to each participant (Appendix 7). They were asked to enter the name, brand and weight of all foods and drinks consumed over the 5-day (3 weekdays + 2 weekend days) period. Participants were asked not to make any changes in their diet during the recording phase. Also, participants were instructed not to include special occasions e.g. a birthday or a holiday. Although a 7-day food record is the gold standard of food intake assessments, a four or five day record is a reasonable compromise for assessing an individual's energy and macronutrient intake as this approach decreases the burden on participants and improves compliance (Willett, 2012). For food and drink consumed outside the home, especially on weekends, participants were asked to record as much detail as possible on the WR sheet (Appendix 7). Upon completing the 5-day food record, participants returned all booklets to the researcher, allowing the researcher to follow up on incomplete sections/information. Data from the food records were entered onto Nutritics 5.0 software (Nutritics LTD, Dublin, Ireland), a database that contains 250,000 foods. Foods consumed by participants that were not in the database were manually entered using manufacturer or restaurant nutritional information. Any item for which it was difficult to find any nutritional information was substituted with the closest matching item. The mean daily intake of the 5-day food record was used to represent the SF value from the food records.

2.2.4 Statistical analysis

Data analysis was conducted to assess FFQ validity. Participants with poor recording or extreme outliers, with values outside the range of ± 2 SD, were excluded. Mean and standard deviations (SD) were calculated for SF intake as provided by the FFQ, and WR for comparison. Normality tests were conducted using the Shapiro-Wilk test. Non-parametric tests were performed using the Wilcoxon signed-rank test to determine differences between means for SF intake from the two dietary assessment methods. Significant differences were indicated by P values less than 0.05. The relationship between SF from FFQs and WR was estimated using Spearman Rho correlation coefficients to assess the strength of association between the two methods. A strong correlation was represented if the correlation coefficients $r \geq 0.50$, a moderate correlation outcome was between 0.20 – 0.49 and a weak correlation if less than 0.20 (Lombard *et al.*, 2015). Intra-class correlation coefficients (ICC) were also calculated as they were better measures of strength of association, when compared to the Spearman Rho correlation. A strong agreement between the tools was reflected by values greater than 0.4 (Gibson, 2005). Analysis of correlation coefficients and ICCs were performed using the bootstrap method. Bootstrapping is a statistical technique that measures the accuracy of sample estimates by resampling a dataset to create many simulated samples (Bland and Altman, 2015). This process calculates standard errors and confidence intervals without any reliance on assumed probability distributions (Bland and Altman, 2015). A cross classification was performed between SF intake from the FFQ and WR to assess whether the FFQ could be used to rank individuals according to their SF intake from the WR. The percentage of participants classified into the same quartile, same or within one quartile and misclassified

quartiles was calculated. Misclassified quartiles were identified when one method was in the lowest quartile while the other method was in the highest quartile.

The Bland-Altman method was also used to assess the agreement between the mean intakes of SF derived from the two different tools. The difference in intake between the two methods (FFQ-WR) was plotted against the mean intake of the two measures $((\text{FFQ} + \text{WR})/2)$ (Flood *et al.*, 2004). The limits of agreement, $\text{mean} \pm 1.96 \text{ SD}$, were used to show how well the two methods agreed, and the average mean of difference indicated whether one method tended to over or underestimate PC intake. Passing and Bablok regression was performed to determine linear model validity between the two methods; *P* values greater than 0.05 were considered as good outcomes (Lombard *et al.*, 2015). Data analyses were carried out using the Statistical Package for the Social Sciences (SPSS; version 25.0) and MedCalc software (version 16.4.3, Ostend, Belgium) was used to perform Bland-Altman and Passing-Bablok regression tests.

The aim was to recruit 100 participants for this study, based on a previous recommendation that a FFQ validation study should be conducted with at least 50–100 participants (Cade *et al.*, 2002). Study power was calculated by G*Power software version 3.0.10. Calculations were based on correlation statistical tests (two-tailed) and effect size of 0.3 with β/α ratio 2. Calculations showed that a 97 participant rate resulted in 88% power in detecting statistically significant correlations between dietary intake of SF from FFQs and WR.

2.3 Results

One hundred and nine participants were enrolled in this study, five withdrew from the study for personal reasons and seven participants were excluded due to poor food

recording (n=3) or extreme outliers (n=4). The results recorded are for 97 participants (29 males, 68 females). The mean age was 33 ± 10 years (Table 2.2).

Table 2.2 Characteristics of participants in the FFQ validation study (n=97)

Characteristics	n	%
Gender		
Male	29	29.9
Female	68	70.1
Age (yrs)		
18 -29	39	40.2
30 -39	34	35.1
40 – 49	12	12.3
50 – 59	10	10.3
60+	2	2.1
Ethnicity		
White	57	58.8
Asian/Asian British	12	12.4
Mixed/Multiple ethnic groups	10	10.3
Arab	15	15.5
Black/African/ Caribbean's /Black British	3	3.1
Smoking		
Smoking –never	80	82.5
Smoker- past	12	12.4
Smoking < 10 per day	4	4.1
Smoking 11-20 per day	1	1
Alcohol consumption*		
Sensible drinking	51	52.6
Hazardous drinking	9	9.3
Harmful drinking	3	3.1
Never	34	35.1
Body mass index BMI		
Underweight	6	6.2
Normal	51	52.6
Overweight	29	29.9
Obese I	9	9.3
Obese II	2	2.1

* Alcohol consumption classified as per the Alcohol Use Disorders Identification Test (AUDIT; Saunders *et al.*, 1993). Scoring between 0-7 points is indicated as sensible drinking, between 8-15 points is indicated as hazardous drinking and between 16-19 points is indicated as harmful drinking.

Over half of participants were white (58.8%). The majority of participants were non-smokers (82.5%), while 52.6% drank alcohol sensibly and 35.1% were non-drinkers. For the BMI, 51% of participants were in the normal weight range.

The mean dietary PC intake is presented (Table 2.3). The average PC intake was approximately 255 mg per day. Although the intake of dietary PC tended to be higher

among men compared to women, there were no significant differences in average intakes between the two groups.

Table 2.3 Dietary PC intake per day (mean $\pm$ SD)*

Group	FFQ PC (mg/d)	<i>P</i> value
All subjects n = 97	255.1 $\pm$ 205.2	
Male n = 29	289.3 $\pm$ 252.2	0.803 [#]
Female n = 68	240.5 $\pm$ 182.0	

*Data are expressed as the mean $\pm$ standard deviation of dietary phosphatidylcholine intake from food frequency questionnaires. [#]Mann-Whitney test was used to compare between male and female mean intake.

The SF mean intake from the WR was 27.9 $\pm$ 10.5 g, which was higher than the SF mean intake from the FFQ (22.6 $\pm$ 13.4 g). Evaluation of differences between the SF mean intake from the two methods, for all participants showed a significant difference ($P < 0.001$; Table 2.4).

Table 2.4 Comparison of SF intake per day from FFQ and WR*

Group	FFQ SF (g/d)	WR SF (g/d)	<i>P</i>
All subjects n = 97	22.6 $\pm$ 13.4	27.9 $\pm$ 10.5	< 0.001
Male n = 29	24.7 $\pm$ 18.7	32.5 $\pm$ 14.1	0.053
Female n = 68	21.6 $\pm$ 10.3	25.9 $\pm$ 8.0	0.002

*Data are expressed as the mean $\pm$ standard deviation of saturated fat intake from each method. Values indicate statistical significance ($P < 0.05$) as analysed by the Wilcoxon signed-rank test.

Correlations between the mean SF intakes derived from the two methods are shown in Table 2.5. The Spearman Rho correlation coefficient for the crude data was weak (r 0.12, P 0.251). Intra-class correlation coefficients improved the correlation factor to r 0.23.

Table 2.5 Correlation coefficients between dietary SF intake from FFQ and WR for all participants*

Group	Correlation coefficient	ICC
All subjects n = 97	0.12	0.23
Male n = 29	0.20	0.23
Female n = 68	0.03	0.12

*Test performed using the bootstrap technique.

In performing cross-classifications, we found that 61% of all participants were in the same or within one quartile, while 39% were misclassified (Table 2.6). Comparing males and females, there were no major differences between the two groups. However, males were classified in the same quartile, more than females.

Table 2.6 Cross-classification of participants by quartiles of SF intake from the FFQ and WR

Group	Same quartile		Same or within one quartile		Grossly misclassified	
	n	%	n	%	n	%
All subjects n = 97	30	30.90	59	60.80	38	39.20
Male n = 29	10	34.50	18	62.10	11	37.90
Female n = 68	20	29.40	41	60.30	27	39.70

Bland-Altman plots showed acceptable agreements between SF intake from FFQ and WR (mean difference -5.4 g, 95% CI -8.52 to -2.28, limits of agreement 25.1 and -36.0). Moreover, the Passing-Bablok regression analysis (Intercept A -10.6684, 95% CI -25.9957 to 0.2000, Slope B 1.1675, 95% CI 0.7294 to 1.7929) showed no systematic differences between the two methods (Intercept A including zero) and there were no proportional differences between the two methods (Slope B included 1 value). However, there was no significant deviations from linearity using the Cusum test for linearity ($P = 0.50$; Figure 2.1).

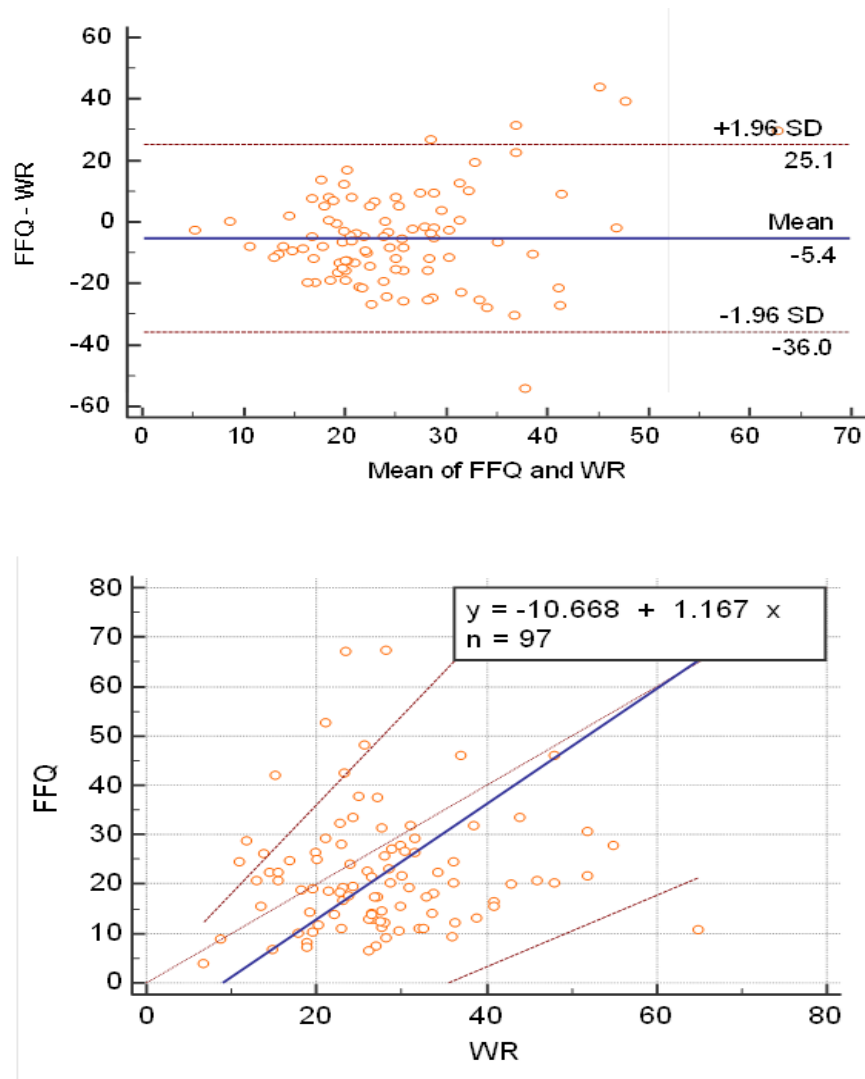


Figure 2.1 Bland-Altman test and Passing-Bablok regression analysis between saturated fat intake from WR and FFQ methods

2.4 Discussion

Developing a valid and comprehensive tool to measure PC intake is necessary, especially when it addresses the relationship between PC intake and the risk of developing CVDs. This study validated a novel 136 item self-administrated FFQ to measure dietary PC intake in the general population of the UK. Currently, there is a gap in determining the PC content in foods, especially when it comes to software that analyses food records, therefore adding another nutrient to the questionnaire was necessary for validation purposes. Saturated fat (SF) was chosen as it has been shown

to have a good level of correlation between FFQ and dietary history (Feunekes *et al.*, 1993). In this study, the dietary intake of SF from two methods (FFQ and WR) showed acceptable levels of agreement using Bland-Altman plots.

The absolute mean intake of SF from WR was higher than similar data derived from the FFQ, which was a reported issue in previous research (Broadfield *et al.*, 2003). Some fats and oils, for example sunflower oil, were not included in the FFQ because of missing PC values in the USDA database. This may in part explain why SF values tended to be higher in the WR when compared to the FFQ.

The mean intake of dietary PC in this study was determined at 255 mg per day. In a recent study, conducted in US adults, the mean dietary PC intake was 209 mg per day (Zheng *et al.*, 2016). PCs are present in a wide range of food sources, but with different concentrations, for example high sources of PC include egg yolk and beef liver while low PC levels are found in fruit and vegetables (Patterson *et al.*, 2008). Besides natural sources, PC is also found as lecithin in processed foods. Although lecithin is widely used as an emulsifier, maximum lecithin levels do not exceed 1% in the final product preparation (Leray, 2014). Thus, PCs consumed by participants from other processed foods, which are not mentioned in the FFQ, should not significantly affect the average mean intake measured in this study. The ICC for SF, between the FFQ and the WR in this study showed weak correlation (r 0.23), yet this result was higher when compared to a previous study (r 0.11; Sauvageot *et al.*, 2013). However, similar validation studies had better SF intake correlations: e.g. r 0.75, 0.69, 0.62, 0.50 for Feunekes *et al.*, 1993; Van Assema *et al.*, 2001; Broadfield *et al.*, 2003; Rohrmann and Klein, 2003, respectively. Correlation is the most common method used to assess FFQ validity and is used in over 90% of validation studies. However, this approach was questioned, as it

only measures the degree to which the two methods are related, rather than measuring agreements between them (Cade *et al.*, 2004). Also, correlation is greatly affected by the study population size and participant characteristics. Therefore, cross-classification into quartiles and Bland-Altman plots are recommended for investigating any agreements between the two methods (Cade *et al.*, 2002). In our study, when using Bland-Altman analysis, no systematic changes were observed in the magnitude of between-measurements (FFQ vs WR) across mean values of SF in both tools. This means there was agreement between FFQ and WR in term of SF intake, regardless of participants' average intake.

The use of Bland–Altman plots for the assessment of FFQ validity and measurement of level of agreement was started to be used in 1993 (Thompson and Margetts, 1993). In a previous validation study of semi-quantitative FFQ in reference to 24 hr recall, Bland-Altman plots shown an acceptable level of agreement between the two methods (FFQ and 24 hr recall) in terms of total fat which agrees with our study results (Dehghan *et al.*, 2012). However, in another study conducted by Christensen *et al.* (2013), Bland-Altman plots showed differences in SF intake, as assessed by FFQ and WR plotted against the mean of both tools.

In the current study, the majority of participants were female (70%), under 50 years of age (87%) and in the normal weight category (52%). From the literature, it has been shown that some factors may affect dietary recall and FFQ validation, for example gender, BMI and season (Fowke *et al.*, 2004; Paalanen *et al.*, 2006).

Gender differences can influence FFQ validation due to differences in food item intake and portion sizes between women and men (Lee *et al.*, 2016). Food intake is usually higher in men than women and portion sizes also differ between the two genders.

Therefore, the importance of using a sex-specific food portion, instead of the standard food portion size needs to be addressed (Cade *et al.*, 2002). In term of FFQ validity, it was found that women were more likely to under-report their food intake while men tend to over-reporting (Johansson *et al.*, 1998). The under-reporting issues in women could be explained by either undereating (Goris and Westerterp, 1999) or under-reporting snack foods (Heitmann and Lissner, 1995). Furthermore, body weight status can affect FFQ validation as under-reporting was a common problem among people with high BMI comparing to normal BMI level (Freedman *et al.*, 2014) as the person attitude toward their own body image can be strongly impact the reporting of food intake (Johansson *et al.*, 1998). More specific, underestimation in recording portion size especially among food types that defined as unhealthy or bad food could be the reason behind under-reporting in higher BMI participants (Lafay *et al.*, 2000). In our study more than half of the participants were females and classified within the normal body weight. However, the impact of these variables on our study results was difficult to assess, due to the inequality of participant numbers in the groups affected by these factors.

Furthermore, seasonal contributions have been fully investigated in previous studies. In a US study (Ma *et al.*, 2006), 593 participants were followed over a one year period to identify the effects of seasonal variation on food intake. Participants were asked to record their 24-h dietary recall, on three random days, five times a year, at baseline, three, six, nine and 12 months. The results showed that daily caloric intake was highest in the Autumn and peaked in early November ($P = 0.05$), while calories from fat intake peaked in early September ($P = 0.038$). Moreover, the percentage of calories from SF was highest between September and December and peaked in early November ($P =$

0.02; Ma *et al.*, 2006). In our study, recruitment occurred between February and November, therefore this period could be a limitation of the study. However, as 75% of participants completed the study between February and August, these seasonal variations might have had negligible impact on FFQ validity.

The measurement of dietary intake is complicated, no methods are free from errors. A key component and a major strength of this validation study was the selection and incorporation of the WR as a reference method. This approach is the first choice for validating FFQs as it is a gold standard in dietary assessment methods (Willett, 2012). The weighed records approach has less correlation errors when compared with other reference methods, such as the 24 hour recall method (Livingstone *et al.*, 1990), but it still has weaknesses. Memory limitation for WR is not a source of error, especially when it is compared to the FFQ. However, delays in recording by some participants may lead to information errors, due to poor memory. Also, writing down and recording food intake may lead to changes in dietary behaviours, food intake may be accurately written/recorded, but it may not represent what was eaten. This could be to minimise the effort of recording by trying to consume more simple food rather than mixed dishes containing many ingredients (Willett, 2012).

The limitations of the current study must be considered. Firstly, we measured SF values from two different databases. For the WR, we analysed SF values using Nutritics software that used McCance and Widdowson 7th edition databases (2015), while for the FFQ, we calculated the SF using the USDA database. The independent coding of FFQ and WR in our study may be a reason for the lower relative validity of our study, when compared with other studies (Van Assema *et al.*, 2001; Broadfield *et al.*, 2003).

Secondly, although the study power reached 88%, it was difficult to detect a strong correlation coefficient between the two methods. A larger sample size may be needed to strengthen these correlations and to reach a statistical significance, even for smaller correlations.

Interest in nutritional epidemiology is high as the impact of diets on chronic diseases, especially CVDs, becomes widely recognised and accepted (Bazzano *et al.*, 2002; Tanasescu *et al.*, 2004). However, methodologies for measuring and analysing diets are still being developed. Better methods, incorporating biological biomarkers should be considered in the future; however, currently, the FFQ is the method most commonly used for large epidemiological studies.

2.5 Conclusions

This study validated a 136-item self-administrated FFQ to measure the dietary intake of PC among the UK population. Validity was assessed by comparing the estimated daily intake of SF from FFQs, with the mean of a 5-day WR approach. Statistical analysis demonstrated that the FFQ can be a useful tool in estimating the intake of dietary PC and may be used in PC epidemiological studies in relation to CVD and other chronic diseases. The results had acceptable levels of relative validity based on Bland-Altman plots; however, in considering previous FFQ limitations, this FFQ cannot be generalised to the UK population as a whole, therefore this FFQ must be tested in larger cohorts.

Chapter 3: Dietary phosphatidylcholine assessed by self-administrated food-frequency questionnaire in relation to cardio-metabolic biomarkers in healthy populations (Study 2)

3.1 Introduction

Hyperlipidaemia refers to abnormal levels of blood lipids and is one of the most frequent risk factors for developing CVD globally (Reiner *et al.*, 2011). PC is a major class of PLs and plays a vital role in the body, as it forms a significant part of the cellular membrane. It is a major component of all lipoproteins (HDL, LDL, IDL, VLDL and chylomicrons), which helps in fat transportation around the body (Cohn *et al.*, 2010). PCs are derived from plant and animal sources (Zeisel *et al.*, 2003). A previous study assessed dietary PC intake and found that the average intake in humans was 209 mg/day (Zheng *et al.*, 2016), however, there is no recommended PC daily intake, which makes it difficult to ascertain if this amount is sufficient for human health.

Heretofore, dietary PC has not been explored intensively in epidemiological studies. This may be due to the establishment and limited food composition database that provides PC content in some common foods (Patterson *et al.*, 2008). Also, it is difficult to address the effects of dietary PC intake on some diseases, as it is present in many food sources not found in the USDA database, e.g. sunflower and coconut oil. The absence of a valid nutritional tool to measure dietary PC intake may be an additional reason for the lack of studies exploring dietary PC. However, to address this research gap, our previous study (Chapter 2) provides a novel valid FFQ to measure dietary PC.

In the literature, the relative importance of PC on health effects is largely inconsistent. Some studies have suggested that PC supplementation can help lower fasting total

homocysteine (tHcy; Olthof *et al.*, 2005) and reduce atherogenic lipoproteins (Høie *et al.*, 2005). On the other hand, a number of studies have suggested that dietary PC metabolism increases the risk of developing CVDs by producing TMAO (Wang *et al.*, 2011; Tang *et al.*, 2013).

From a nutritional aspect, very little is known about PC and a much debated question is whether dietary PC intake can help prevent CVDs. Therefore, this study investigated dietary PC intake as assessed by a self-administrated FFQ, in relation to lipid and cardio-metabolic markers, including; LDLC, HDLC, TC, TAGs and Lp(a). Also, this study determined the relationship between PC dietary intake and PC concentrations in the blood. This study may help establish dietary reference values (DRVs) for PC.

3.2 Methodology

3.2.1 Study participants and baseline characteristics

A total of 109 participants with a mean age of 33 ± 10 years, were recruited through study advertisements. Inclusion criteria included healthy participants (men and women) aged between 18 to 65 years. Pregnant women and people with medical conditions such as heart disease, diabetes and hypertension and renal or liver disease were excluded. In previous studies (Tang *et al.*, 2013; Zheng *et al.*, 2016), the association between dietary PC and CVD risk was investigated in populations with traditional risk factors of developing CVD, such as history of heart failure and CVD, hypertension, diabetes mellitus and renal impairment function (Tang *et al.*, 2017). In this research study, it was hypothesized that dietary PC intake is related to cardio-metabolic biomarkers, independent of traditional risk factors. Thus, the relationship

was assessed in individuals at low risk of CVD, to assess dietary PC as an independent risk factor.

Ethical approval was been obtained from UREC at OBU (Appendix 1a). For each participant, written informed consent was obtained before study participation (Appendix 3a).

3.2.2 Study design

This part of the study is a continuation of the previous study (Chapter 2). The study procedure was explained to the participant (Appendix 2a) and if they agreed to participate a consent form was signed (Appendix 3a). Participants were required to complete a health questionnaire that contained information about their smoking habits, medical conditions and ethnicity (Appendix 4a). Participants also completed the long version of the validated international physical activity questionnaire (IPAQ; Craig *et al.*, 2003) which measures physical activity level over the previous seven days (Appendix 6a). This questionnaire determined participant average physical activity patterns. The alcohol consumption questionnaire, (AUDIT) identified participant drinking levels (Appendix 5). Drinking habits were defined as: sensible drinking, hazardous drinking, harmful drinking or possible dependence. This questionnaire is validated to be used to assess the level of risk to alcohol harm by the Public Health England (2017) and it was important to ascertained participant average alcohol consumption and ensured this consumption was not classified as heavy, which could affect lipid profile levels (Wakabayashi, 2014).

Body composition was assessed by measuring height, weight, body fat percentage and waist and hip circumference. Weight and body fat percentages were measured by a segmental body composition analyser (Tanita BC-418 MA, Illinois, USA). Height was

measured using a stadiometer tape (Seca Ltd, Birmingham, United Kingdom). Waist and hip circumferences were measured using a non-stretchable tape (Seca Ltd, Birmingham, United Kingdom). Waist circumference was measured at the narrowest point above the navel, and hip circumferences were measured at the widest circumference of the hipbones. Data were recorded to the nearest 0.1 cm. Participants were asked to wear light clothes on the test day. Measurements were recorded without shoes and outer clothes.

3.2.3 Dietary assessments

The FFQ assessed participant PC intake over the past year (Appendix 8). One hundred and thirty-six items were included, which was a modified and adjusted version of a previously validated questionnaire (Salvini *et al.*, 1989). A validation study, assessing the reliability of this FFQ, showed an acceptable level of agreement (Chapter 2). Therefore, it was used in this study.

3.2.4 Blood sampling

3.2.4.1 Blood collection and storage

A venous blood sample was collected from each participant in two 4 ml plain blood tubes (BD Vacutainer, Belliver Industrial Estate, Plymouth, UK) using a 21G needle (BD Vacutainer, Plymouth, UK). Blood was collected from the superficial vein which lies within the anterior cavity of the elbow. Blood taking was performed using WHO (2010) guidelines for venepuncture blood drawing. After collection, blood samples were allowed to clot for at least half an hour at room temperature (Tolonen *et al.*, 2002). After this period, they were centrifuged to separate serum from blood cells. For lipid profile biomarkers and Lp(a) analyses, samples were centrifuged at 3000 x g for 15 minutes at 15°C. For PC analyses, blood samples were centrifuged at 2000 x g for 10

minutes at 4°C. The serum was collected by pasteur pipette in 1.5 ml aliquots and stored at different temperatures depending on the marker; for TCs, TAGs and LDLC, serum was stored at +4°C, for Lp(a) analysis, serum was stored at -20 °C, and for HDLC and PC, serum was frozen at -80°C. Samples were stored according to Randox manufacturers' guidelines (Tietz, 2006).

TCs, TAGs, LDLC and PC analyses were conducted at Oxford Brookes University laboratory, while HDLC and Lp(a) were measured at Randox Health Laboratory, London, UK.

3.2.4.2 Blood analysis

Analyses of TC, TAGs and LDLC were performed using the Randox kit; CH-200 cholesterol, TR-210 triglycerides and CH-2657 direct LDL-cholesterol on an Rx Monza, a semi-automated analyser (Randox Laboratories Ltd, Crumlin and Antrim, UK), according to manufacturer's instructions. Blood analysis of TC, TAGs and LDLC was performed within four days of sample collection.

Equipment calibration was carried out daily, while quality control (QC) was performed weekly according to manufacturer recommendations. All reagents and samples were acclimatised to room temperature, prior to analysis. The Rx Monza was calibrated using the CHOL CAL, TAGs CAL and direct LDL/HDL cholesterol calibrator for TC, TAGs and LDLC, respectively. Analyses for TC and TAGs were similar in procedure. TC was measured using the cholesterol oxidase method, while TAGs were assayed using the lipase/GPO-PAP no correction method.

Approximately 10 µl serum was mixed in a 5 ml cuvette (Neolab Migge GmbH, Heidelberg, Germany) with 1000 µl cholesterol or triglyceride reagent. The cuvette was then incubated on a dry block thermostat (Grant Instruments Ltd, Cambridge, United

Kingdom) at 37 °C for 5 minutes. After incubation, the cuvette was inserted into the Rx Monza flow cell holder, for assay. The detectable range for TC is 0.345 – 17.0 mmol/L, while for TAGs, it is 0.26 – 13.42 mmol/L.

LDLC was measured using the direct clearance method. Approximately 12 µl blood serum was mixed in a cuvette with 900 µl LDLC reagent 1 and incubated at 37°C for 5 minutes. After this, 300µl LDLC enzyme reagent 2 was added to the cuvette and assayed immediately. The detection range for LDLC was 0.53 – 22.8 mmol/L.

Serum samples to detect HDLC and Lp(a) were analysed at Randox Health laboratory in London, using a Randox Daytona analyser. For HDLC assay, the direct clearance method was used and for Lp(a), the immunoturbidimetric method was used. Samples were sent to Randox Health Laboratory to be analysed as HDLC and Lp(a) kits required Randox Daytona analyser that was not available at OBU. Blood analysis of HDLC and Lp(a) was performed 11 months after sample collection.

A phosphatidylcholine assay was performed using a STA-600 phosphatidylcholine assay kit (Cell Biolabs, San Diego, USA). Analysis was performed using a fluorescence microplate reader, using 560 nm excitation and 590nm emission wavelengths (Infinite F200 PRO, Tecan Group Ltd, Männedorf, Switzerland). Serum samples were thawed at room temperature and reagents were prepared as follows: (1) 25 ml assay buffer was diluted with 225 ml deionised water, (2) 1 ml standard diluent was diluted with 9 ml deionised water, (3) the reaction reagent was prepared by diluting 25 µl choline oxidase, 10 µl HRP, 50 µl fluorescence probe and 15 µl phospholipase D together. Finally, assay buffer was added to the prepared reaction agent solution up to 5 ml. The reaction reagent solution was prepared in a dark room as it was light sensitive. Once

serum samples were completely thawed, they were vortexed to mix and appropriate standards were prepared (Figure 3.1).

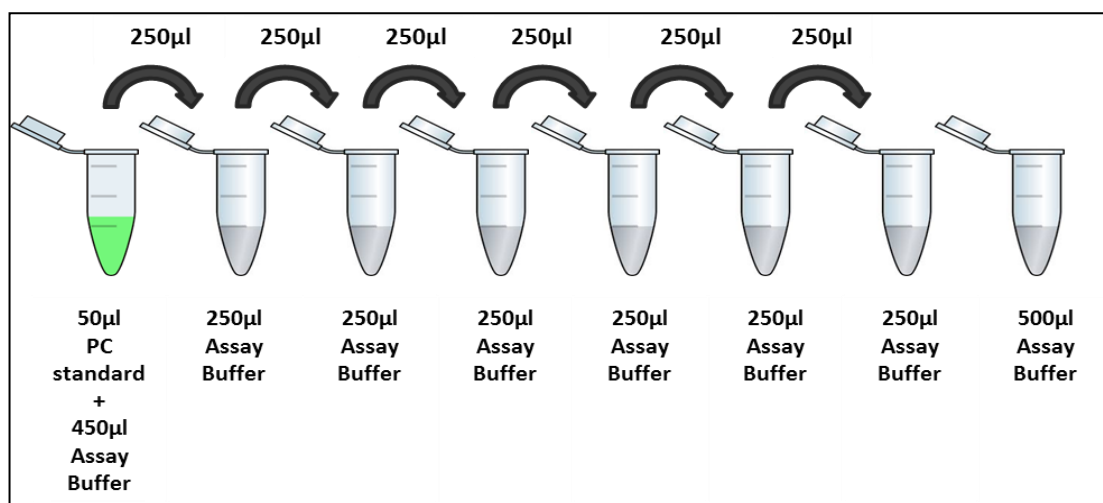


Figure 3.1 Serial dilutions of phosphatidylcholine standards

A 96-well plate was used for PC analysis. Columns one and two were used for standards, whereas columns 3 – 12 were for the serum samples. Wells G12 and H12 were kept blank for standardisation. Standards and samples were assayed in duplicate. Approximately, 10 µl of PC standards were added to columns one and two, while 10 µl of sample was added, in duplicate, to wells in columns 3 – 12. Next, 100 µl of reaction reagent was added to each well and mixed thoroughly by moving the plate gently, right and left. The plate was then covered by clear sealing film and incubated for 60 minutes in a dark incubator at 37°C (Mettler GmbH + Co. KG, Schwabach, Germany). After this period, the plate was read and PC concentrations were calculated based on the PC standard curve. Blood analysis of PCs was performed twice at four and eight months after sample collection.

3.2.5 Statistical analysis

Data analysis was carried out using the Statistical Package for the Social Sciences (SPSS) software for windows, version 25.0 (IBM corp., Armonk, NY, USA) to assess correlations between PC dietary intake and cardio-metabolic biomarkers, including TCs, TAGs, LDLC, HDLC, Lp(a) and PC blood serum. Descriptive statistics were used to calculate the mean and SD to describe study participants baseline characteristics. Normality tests were conducted using the Shapiro-Wilk test. Data were not normally distributed, thus non-parametric tests were carried out. The relationship between dietary PC intake and blood biomarkers was measured using Spearman rho correlation coefficients. Strong correlations were represented if the correlation coefficient r was ≥ 0.50 , medium outcomes were between 0.30 – 0.49 and they were weak, if less than 0.29 (Lombard *et al.*, 2015). Statistical significance was set at $P < 0.05$. Correlations between dietary PC intake and cardio-metabolic biomarkers were analysed based on age, BMI and gender. Splitting the groups according to age was based on NHS recommendations; it was important to screen every person above 40 years for CVD risk (NHS, 2018). Gender differences and CVD risks have been extensively explored in the literature (Maas and Appelman, 2010), therefore it was important for this study to explore these differences. Obesity is also a risk factor in CVD (Lavie *et al.*, 2009), thus this was another variable to consider in exploring differences between study participants, based on their BMI classification.

Sample size was calculated based on a previous study of random sample size of healthy and high risk group (Alkerwi *et al.*, 2010), where the recruiting of 141 participants ensured a statistical absolute precision of $d = 0.02$, for the estimation of risk developing CVDs, at 95% confidence intervals. However, in this study only 101

participants were recruited. This sample size had a 91% power in detecting a relationship between PC dietary intake with cardio-metabolic biomarkers, based on correlation t-tests and an effect size of 0.3 with 2 β/α ratio (one-tail). Power and sample calculations were carried out using G* power software version 3.0.10.

3.3 Results

A total of 109 participants took part in this study. Five participants withdrew for personal reasons, and three were excluded due to difficulties in blood collection. Therefore, data and results are shown are for 101 participants. The distribution of main baseline characteristics of the study population is presented in Table 3.1. More than half of participants were female (68.3%), white (57.4%) and non-smokers (82.2%). Most participants were active with high IPAQ scores and were classified with normal body weights.

Table 3.1 Baseline characteristics of study participants (n = 101)

Characteristics	Mean $\pm$ SD
Age, y	33 $\pm$ 10
Gender	
Male <i>n</i> (%)	32 (31.7)
Female <i>n</i> (%)	69 (68.3)
Ethnicity	
White <i>n</i> (%)	58 (57.4)
Asian/Asian British <i>n</i> (%)	12 (11.9)
Mixed/Multiple ethnic group's <i>n</i> (%)	9 (8.9)
Arab <i>n</i> (%)	19 (18.8)
Black/African/Caribbean/Black British <i>n</i> (%)	3 (3.0)
Weight, kg	69.1 $\pm$ 12.9
Height, cm	166.7 $\pm$ 9.4
Body mass index BMI	24.9 $\pm$ 4.2
Underweight <i>n</i> (%)	6 (5.9)
Normal <i>n</i> (%)	51 (50.5)
Overweight <i>n</i> (%)	32 (31.7)
Obese I <i>n</i> (%)	9 (8.9)
Obese II <i>n</i> (%)	3 (3.0)
Waist circumference, cm	82.6 $\pm$ 11.4
IPAQ	
Low <i>n</i> (%)	4 (4.0)
Moderate <i>n</i> (%)	46 (45.5)
High <i>n</i> (%)	51 (50.5)
Alcohol consumption	
Sensible drinking <i>n</i> (%)	52 (51.5)
Hazardous drinking <i>n</i> (%)	10 (9.9)
Harmful drinking <i>n</i> (%)	3 (3.0)
Never <i>n</i> (%)	36 (35.6)
Smoking	
Smoking –never <i>n</i> (%)	83 (82.2)
Smoker- past <i>n</i> (%)	12 (11.9)
Smoking < 10 per day <i>n</i> (%)	5 (5)
Smoking 11-20 per day <i>n</i> (%)	1 (1)
Medical history	
Healthy <i>n</i> (%)	85 (85.5)
Hypothyroidism <i>n</i> (%)	3 (3)
Hypothyroidism/ hyperparathyroidism <i>n</i> (%)	1 (1)
Asthma <i>n</i> (%)	4 (4)
Asthma/ Crohn's disease <i>n</i> (%)	1 (1)
Gout <i>n</i> (%)	1 (1)
Poly cystic ovary syndrome <i>n</i> (%)	3 (3)
Celiac disease <i>n</i> (%)	1 (1)
Depression <i>n</i> (%)	2 (2)
Blood pressure, mm Hg	
Systolic	113.9 $\pm$ 12.2
Diastolic	77.47 $\pm$ 8.36
Blood biomarkers	
Total cholesterol, mmol/L	4.4 $\pm$ 0.9
Triacylglycerols, mmol/L	1.1 $\pm$ 0.8
LDL cholesterol, mmol/L	2.4 $\pm$ 0.7
HDL cholesterol, mmol/L	1.4 $\pm$ 0.4
Lipoprotein (a), mg/L	188.0 $\pm$ 229.6
Phosphatidylcholine, mg/dL	7.4 $\pm$ 1.7
Dietary intake	
Phosphatidylcholine intake, mg/d	238.7 $\pm$ 174.3

Within participants, the mean dietary intake of PC was 293.5 mg/day for men, and 213.3 mg/day for women. The top ten food items rich in PC are listed in the FFQ, and the quantity per serving versus the top ten foods consumed, with associated PC content, is presented in Table 3.2.

Table 3.2 Top ten phosphatidylcholine food sources versus the most consumed food by study participants

Ranking	Top foods rich in PC ^a		Top food consumed / week	
	Food	content (mg/serving)	Food	content (mg/serving)
1	Liver, beef	212.5	Banana	0.4
2	Liver, chicken	178.4	Wheat bread	0.9
3	Eggs, whole	121.8	Egg, whole	121.8
4	Soybean	120.9	Onion	1.7
5	Egg, yolk	107.1	Broccoli	32.8
6	Hot & sour soup	86.21	Cheese, cheddar	2.1
7	Bacon, pork	73.1	Carrots	1.1
8	Steak, beef	60.35	Apple	3.9
9	Shrimp	56.95	Sweet pepper	1.6
10	Loin, Pork	52.7	Crisps	2.1

^a USDA, 2008

Table 3.2 shows that participants were consuming foods low in PC. The top food consumed by the majority of participants, were bananas, with only six participants responding as never consumed. Whole egg, which is a primary source of dietary PC, was ranked as the third top food consumed by study participants. Higher intakes of dietary PC were not significantly correlated with inflammatory biomarkers, except for TAGs ($P = 0.017$; Table 3.3).

Correlation analyses were performed using different models to avoid factors that overlap such as sex, age and BMI. In Table 3.3, TAGs showed a significant negative correlation ($P = 0.017$) in the female group, but not in the male group. In addition, HDLC showed a non-significant weak positive correlation in the female group. In

addition, TC, LDLC and Lp(a) showed very weak correlations. However, in the male group no significant correlation was recorded.

Table 3.3 Correlation of dietary PC intake with cardio-metabolic biomarkers, according to gender

Variables	All subjects <i>n</i> =101	<i>P</i>	Male <i>n</i> =32	<i>P</i>	Female <i>n</i> = 69	<i>P</i>
TC	-.059	0.561	-.172	0.346	.008	0.947
TAGs	-.238*	0.017	-.147	0.423	-.292*	0.015
LDLC	.049	0.672	.113	0.538	.000	0.999
HDLC	.106	0.298	.010	0.955	.215	0.076
Lp(a)	.068	0.502	.140	0.443	.019	0.879

Sig values *P* <0.05

Table 3.4 shows the interrelationship between PC intake and lipid profile, according to age. Participants were split into two age groups; ≥ 40 years and < 40 years of age. The relationship between PC consumption and Lp(a) was stronger in the ≥ 40 group when compared to the < 40 group, however, this was not significant (*P* = 0.47). HDLC levels showed a non-significant correlation with dietary PC intake in both groups (*P* = 0.535).

Table 3.4 Correlation of dietary PC intake with cardio- related biomarkers, according to age

Variables	Age < 40 <i>n</i> =78	<i>P</i>	Age $\geq$ 40 <i>n</i> =23	<i>P</i>
TC	-.100	0.383	.083	0.707
TAGs	-.187	0.101	-.339	0.114
LDLC	.068	0.555	.066	0.764
HDLC	.117	0.307	.267	0.218
Lp(a)	.024	0.837	.229	0.293

In the model according to BMI, participants were divided into two groups. The first group contained participants with BMIs less than 25kg/m², which included underweight and individuals of normal weight. The other group included participants with BMIs ≥ 25 kg/m², which included overweight and obese individuals. Table 3.5 shows correlation coefficients according to BMI.

Table 3.5 Correlation of dietary PC intake with cardio- related biomarkers, according to body mass index

Variables	BMI < 25 n= 57	<i>P</i>	BMI ≥ 25 n= 44	<i>P</i>
TC	.120	0.357	-.281	0.065
TAGs	-.152	0.257	-.336*	0.026
LDLC	-.019	0.890	.107	0.491
HDLC	.218	0.103	-.047	0.764
Lp(a)	.062	0.649	.065	0.677

Sig values *P* <0.05

There was a significant negative correlation in the BMI ≥ 25 group for TAGs (*P* = 0.026) and a weak negative correlation (*P* = 0.065) for TC in the same group. LDLC also showed a non-significant weak positive correlation in the overweight and obese group (*P* = 0.491). HDL cholesterol showed a non-significant weak positive correlation in the normal weight group. Lp(a) displayed no correlations in both groups.

The second objective of this study was to examine whether PC dietary intake affected PC serum concentrations. Table 3.6 shows correlations between PC intake and PC concentrations in serum. Overall, there were no significant relationships in any groups, as shown by weak correlation coefficients.

Table 3.6 Correlation of dietary PC intake with PC serum concentrations¹

Groups	Dietary PC intake	PC serum concentration	Correlation coefficient	<i>P</i>
All subjects (n= 97)	238.7 ± 174.3	7.39 ± 1.73	0.082	0.424
Male (n= 32)	293.5 ± 258.7	7.19 ± 1.49	-0.101	0.584
Female (n= 65)	213.3 ± 110.2	7.49 ± 1.85	0.196	0.117
Age < 40 (n= 75)	250.2 ± 175.7	7.40 ± 1.64	0.061	0.605
Age ≥ 40 (n= 22)	199.8 ± 167.5	7.37 ± 2.05	0.266	0.232
BMI < 25 (n= 55)	237.5 ± 155.9	7.31 ± 1.69	0.035	0.798
BMI ≥ 25 (n= 42)	240.3 ± 197.5	7.51 ± 1.79	0.178	0.259
Normal waist circumference (n= 79)	232.6 ± 157.5	7.38 ± 1.71	0.118	0.301
High Risk waist circumference*(n= 18)	267.0 ± 240.9	7.44 ± 1.85	-0.088	0.729

¹Data are presented as mean ± SD. The Spearman rho correlation coefficient test assessed correlations between dietary PC intake and serum PC levels. *Disease risk relative to waist circumference is defined for men >102 cm and women >88 cm (WHO, 2008).

3.4 Discussion

The risk of developing a CVD can be influenced by either genetic or environmental factors (Elder *et al.*, 2009). High levels of total blood cholesterol and TAGs are associated with cardiovascular risk (Lusis, 2000), however, there is limited data on the pathogenesis of PLs in CAD. In this study, addressed the relationship between PC dietary intake with cardio-metabolic biomarkers, including TC, TAGs, LDLC, HDLC and Lp(a). The PC dietary intake was assessed using an FFQ shown to have good validity levels with reference to a 5-day WR (Chapter 2).

The mean PC dietary intake in this study was 255 mg per day. In a previous study, conducted in a US population (Zheng *et al.*, 2016), the mean PC dietary intake was 209 mg per day. Currently, there is no recommended daily intake of PC which is challenging as there is no way to estimate if this level of PC consumption is sufficient for human health.

This study determined a significant negative correlation between the intakes of dietary PC and TAG levels. One of the main roles of PC synthesis is stimulation of hepatic VLDL secretion, which reduces the accumulation of TAGs in the liver (Yao and Vance, 1988). The relationship of dietary PC with liver lipoprotein metabolism has been explored previously in animal studies, Buang *et al.* (2005) found that dietary PC, when fed to rats for 10 days, was significantly associated with a reduction in hepatic TAG accumulation and reduced TAG serum levels. Dietary PC played an important role in the regulation of lipogenesis; synthesising TAGs by affecting the enzymes involved in TAGs synthesis (malic enzyme, glucose-6-phosphate dehydrogenase and fatty acid synthase). Similarly, it enhance fatty acids β -oxidation through the activation of carnitine palmitoyltransferase; an enzyme responsible for transferring the acyl group

of long-chain fatty acyl coenzyme-A from Co-A to l-carnitine (Buang *et al.*, 2005). Furthermore, animal studies have proposed that dietary PC plays a role in reducing serum and hepatic lipid accumulation by reducing hepatic TAGs synthesis and decreasing the enzymes responsible for cholesterol esterification in the liver namely, Acyl-CoA: cholesterol acyltransferase (ACAT; LeBlanc *et al.*, 2003; Wat *et al.*, 2009; Ide *et al.*, 2014). However, controlled human trials are required to substantiate the role of PLs, in particular PC, in TAG synthesis.

Moreover, weak correlations have been observed between dietary PC intake and HDLC levels. This finding was also reported by Detopoulou *et al.* (2008) who found that individuals with higher dietary choline intake (an essential nutrient derived mainly from PC) had higher HDLC levels. The relationship between dietary PC and HDLC concentrations was explained in an early study (Zierenberg and Grundy, 1982) where they examined the metabolic fate of dietary PC in men. It was found that dietary PC quite rapidly incorporated into plasma HDL particles after ingestion. The high appearance of PC in plasma HDL can be understood by the direct secretion of PC into nascent HDL derived from intestine (Blesso, 2015). The importance of PC content in HDL has been shown to be a major factor in its ability to accept cholesterol from peripheral cells during the reverse cholesterol transport (Yancey *et al.*, 2000).

Few studies have reviewed the association between dietary choline; an essential nutrient that derived mainly from PC (Patterson *et al.*, 2008) and the risk of CVD. In a prospective study in women, Dalmeijer *et al.* (2008) investigated the relationship between dietary intake of choline, as estimated by FFQ, with CVD risks. Although, results shown that there was no significant relationship with lipid levels (TC, HDLC or LDLC), a high intake of dietary choline was negatively associated with low levels of

serum fasting tHcy; an amino acid that produced naturally from the demethylation of dietary methionine that is found mainly in animal protein sources. Hyperhomocysteinemia, high levels ($> 15 \mu\text{mol/L}$) of tHcy, can be caused by either genetic mutation or environmental factors (Faeh *et al.*, 2006) have been recorded to have a significant correlation with CVD (Ganguly and Alam, 2015).

The mechanism behind the decrease in fasting tHcy levels through choline (PC) is not fully understood. However, this can be explained by the fact that when exogenous PC is increased, the endogenous synthesis of PC via the PEMT pathway is reduced, which in turn may cause a reduction of tHcy levels (Noga *et al.*, 2003c). Approximately 30% of the PC in the body is synthesized through the PEMT pathway, through sequential methylation of PE. As a result of this process, a three homocysteine molecule for each PC molecule is generated (Reo *et al.*, 2002). However, the exact mechanism behind the effect of choline on lowering homocysteine remains unknown.

Recent evidence has shown that dietary PC metabolism can increase the risk of CVD by producing TMAO (Tang *et al.*, 2013). Thus, one aim of this study was to investigate correlations between dietary PC intake and Lp(a) levels. Lipoprotein(a) is a large lipoprotein particle rich in cholesterol, phospholipids, TAGs and Apo(a) and Apo B100. Lp(a) is known to be an independent risk factor for CVD (Nordestgaard *et al.*, 2010). In the current study, it was difficult to detect correlations between dietary PC intake and serum Lp(a) levels.

Previous studies have shown a strong relationship between high levels of Lp(a) and heart disease (Danesh *et al.*, 2000; Kamstrup *et al.*, 2008), believed to be caused by different mechanisms. One such mechanism suggests Lp(a) increases plaque sizes in artery walls, leading to inflammation and increases in vascular smooth muscle cells,

leading to the narrowing of arteries, or atherosclerosis (Fan and Watanabe, 2003). In term of artery wall accumulation, Lp(a) may be more detrimental than LDLC particles, due to the presence of Apo(a) sticky particles (Nielsen *et al.*, 1996). Another explanation suggests that Lp(a) triggers artery blockages by interrupting clotting mechanisms, increasing the risk of heart attack. It is believed that Apo(a) is similar to proteins involved in clotting, forming links between lipids and the coagulation system by preventing fibrin breakdown, the insoluble protein involved in blood clotting. The risk of developing CVD increases in patients with Lp(a) levels greater than 500 mg/L (Nordestgaard *et al.*, 2010).

Levels of Lp(a) are normally not affected by changes in diet, they tend to be influenced by polymorphisms in the Apo(a) gene (McCormick, 2004). However, there is evidence that dietary fat can affect Lp(a) levels. In a 1999 study, Marcovina *et al.* documented levels of Lp(a) in two population from Tanzania - one population comprised Bantu fisherman and the other group were vegetarian. The study documented significantly higher Lp(a) levels in the vegetarians group when compared to the fisherman ($P < 0.001$). This observation coincided with an inverse relationship between Lp(a) levels and plasma omega-3 PUFA ($P = 0.010$) that endorses that fish intake rich in omega-3 PUFA can be responsible of lowering Lp(a) levels (Marcovina *et al.*, 1999). Phosphatidylcholine consists of two fatty acid tails. The most abundant fatty acid compositions in the PC molecule are omega-3 fatty acids e.g α -linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA). Thus, it was believed that PC may be significantly correlated with lower levels of Lp(a). However, no significant relationship ($P = 0.086$) was observed between serum PC levels and serum Lp(a) in this study.

Another study was performed in hyperlipidaemic participants where their diet was supplemented with almonds (Jenkins *et al.*, 2002). The study observed significant reductions in Lp(a) levels ($P = 0.034$), which suggested that monounsaturated fats can help lower Lp(a) levels (Jenkins *et al.*, 2002). In keeping with these observations, a study in 62 healthy obese patients, treated with a low-calorie diet for six weeks, recorded no changes in Lp(a) levels, but significant reductions ($P < 0.05$) were observed in participants pre-treated with high levels of Lp(a) (Kiortsis *et al.*, 2001). These studies support the suggestion that Lp(a) can be affected by diets. However, the difficulty in detecting correlations between the consumption of dietary PC and increasing Lp(a) levels in this study might be due to study design. Firstly, there was no interventional treatment of dietary PC, and secondly it could be due to participant health characteristics, as the majority of participants were under 40 and their mean Lp(a) levels were 188 mg/L, which is not considered high risk. Thus, it was difficult to detect any significant statistical correlations between Lp(a) and PC dietary intake for these reasons.

To our knowledge, only one epidemiological study has investigated the effects of dietary PC intake on CVD risk. A prospective cohort study (Zheng *et al.*, 2016) conducted between 1980 and 2012 in approximately 123,000 individuals, found that non-diabetic participants in the highest quintile of dietary PC intake, had a 9% increase in all-cause mortality risk, and a 19% increase in CVD specific mortality risk, when compared to those in the lowest quintile. However, the study suggested that dietary PC intake was related to a CVD prognosis, more than the development of CVD (Zheng *et al.*, 2016).

These data have been supported by previous studies suggesting that PC intake promotes the development of CVD by producing TMAO molecules as a result of dietary PC catabolism (Wang *et al.*, 2011; Tang *et al.*, 2013). When dietary PC is hydrolysed by intestinal microbes, it produces TMA gas. This gas is absorbed and promptly metabolised by hepatic flavin monooxygenases to form TMAO. A number of studies have found that TMAO contributes to the risk of developing atherosclerotic plaques, by enhancing cholesterol clearance from the liver and its interactions with macrophages and foam cells (Wang *et al.*, 2011). Given this association between serum TMAO levels and the development of atherosclerosis, new recommendations could be proposed in considering TMAO levels as therapeutic targets in lowering CVD risks. However, before confirming this association, these findings must be replicated in various populations as most studies have been conducted in U.S populations (Wang *et al.*, 2011; Tang *et al.*, 2013; Senthong *et al.*, 2016).

In our study, it was difficult to assess whether dietary PC was positively or negatively associated with CVD. Our results showed no relationship between dietary PC and Lp(a) levels. This may have been due to the study population characteristics; there were no apparent risk factors for CVD as mean atherogenic lipoprotein levels (Lp(a), TC, LDLC, TAGs) were normal. Also mean HDLC levels were normal. Moreover, majority of study participant's enjoyed physical activities and had normal body weights and alcohol consumption levels. Although Zheng *et al.* (2016) found significant positive relationships between dietary PC and CVD related mortality, the study was limited in that it did not use a valid FFQ, specialised for measuring dietary PC intake. Also, the study noted that people in the higher quartile of PC intake, were more likely to have a

higher BMI which may be correlated with unhealthy dietary and lifestyle habits e.g. sedentary lifestyle and smoking (Zheng *et al.*, 2016).

The second aim of this study was to assess the impact of dietary PC intake on the serum PC concentrations in the blood. In this study the mean serum PC levels for study participants was 7.4 ± 1.7 mg/dL which fall in the normal range of healthy individuals that vary between 6.9 – 9.7 mg/dL (Sutter *et al.*, 2016).

Overall, there were no correlations between dietary PC intake and PC concentrations in blood. However, there was a weak positive correlation for participants aged ≥ 40 years. The assessment of a specific biomarker, as an indicator of dietary intake, is subject to an accurate assessment of dietary intake. In this study, dietary PC intake was assessed by self-administrated FFQ (Chapter 2). The FFQ was limited in terms of the low numbers of PC containing foods commonly consumed in the UK. There were limited food sources in the database which made it difficult to accurately measure PC consumption. In addition, obtaining accurate dietary information reflecting long term intake was difficult. Ideally, a duplicate diet collection would be made for the period that the lipid pool is reflecting.

Moreover, it is important to consider factors that influence the fatty acid composition of blood lipids, for example age, gender, smoking and exercise. In 1997, Crowe *et al.* (2008) reported positive associations between age and plasma PL ($P < 0.001$) levels in men ($n = 1079$) and women ($n = 1337$), after adjusting for ethnicity, BMI and smoking. Higher PL levels linked with age-related changes in $n-3$ long chain PUFA was reported in previous studies (Dewailly *et al.*, 2001a; Dewailly *et al.*, 2001b) and is suggested to reflect changes in fish consumption with age (Dewailly *et al.*, 2001a).

Gender differences can affect serum PC concentrations in blood by influencing the fatty acid composition of blood lipids. In this study, no significant differences were observed between mean PC serum levels in men (7.5 mg/dl $\pm$ 1.85) compared to women (7.2 mg/dl $\pm$ 1.49); however, in cross-sectional studies, it has been reported that women have higher PL plasma levels than men (Crowe *et al.*, 2008). It is suggested these differences are caused by hormonal effects on the biosynthesis of *n*-3 long chain PUFA. Results from previous studies have indicated that pre-menopausal women have greater capacity to convert 18:3 *n*-3 to 22:6 *n*-3, therefore differences in *n*-3 fatty acid composition between males and females may be explained by sex hormone influences over and above the diet. These 18:3 *n*-3 to 22:6 *n*-3 conversion differences between men and women were attributed to oestrogenic effects in up-regulating delta-6 desaturase, which is a rate-limiting step in this pathway; however, dietary intake of *n*-3 fatty acids were not controlled during the study period (Burdge *et al.*, 2002).

Furthermore, data from the MRFIT study demonstrated that smoking was associated with consistently higher levels of 16:1 *n*-7 and 18:1 *n*-9 fatty acids and lower 22:6 *n*-3 levels (Simon *et al.*, 1996). Cambien *et al.* (1988) also support this finding demonstrating that cigarette smoking was positively associated with 16:1 *n*-7 and 18:1 *n*-9 in levels and negatively associated with 22:6 *n*-3 levels.

The effect of exercise on adipose tissue fatty acid composition and blood lipids was extensively reviewed in the literature (Nikolaidis and Mougios, 2004). Helge and colleagues (2001) investigated the effect of exercise on muscle PL fatty acid composition. Seven healthy male subjects underwent muscle biopsies from the vastus medialis in both legs after days 0 and 4 and four weeks of leg muscle endurance training. After four weeks of training, there were significant increases (between 6%

and 18%) in 18:1 n7, 18:1 n9, 22:6 n3, total MUFA and PUFA levels in skeletal muscle PL in the trained leg compared to the untrained leg. Thus, it would appear that the duration and intensity of exercise modulates the fatty acid composition of muscle PL.

3.5 Study limitations

A limitation of this study relates first to the validity of the FFQ used in this study. Although, validity was examined in a previous study (Chapter 2), there was a limitation of the FFQ in terms of the limited in numbers of food containing PC that are commonly consumed in the UK, (e.g. sunflower and coconut oil). Also, in this study it was difficult to observe any correlations between the consumption of dietary PC intake and Lp(a) levels. This could be limited due several reasons. Firstly, there was no interventional treatment of dietary PC in this study. Secondly, it could be because the mean Lp(a) levels were 188 mg/L for all participants, which is not considered as high levels. Only eight participants were classified in the high CVD risk group. If there was a variation in Lp(a) levels among study participant, there might be a potential to detect a correlation between dietary PC and Lp(a).

3.6 Conclusions

This study has been the first to investigate the relationship between dietary PC intake and cardio-metabolic related biomarkers, in healthy individuals in the UK. Additionally, this study examined the effects of dietary PC on PC serum levels in blood. The results showed a significant negative correlation between PC dietary intake and TAGs levels, however, there was no significant correlation between PC dietary intake and atherogenic lipoproteins or HDLC. Moreover, the concentration of PC in blood appeared to be unaffected by PC dietary intake. The majority of participants were

under 40 years old and free from serious conditions, which may make these findings less generalised to the UK population. A future study could assess the long-term effects of dietary PC against cardio-metabolic biomarkers and TMAO production among a bigger sample size and diverse group, with different ages. Greater efforts are required to understand the absorption pathways of dietary PC, and to develop a food composition database that reflects PC content in common UK foods.

Chapter 4: The effect of phosphatidylcholine supplementation on lipid profile levels among mild hyperlipidaemia individuals (Study 3)

4.1 Introduction

Globally, CVD is the biggest cause of death (WHO, 2017). Coronary artery disease (CAD) is a CVD that affects blood vessels in the heart. Dyslipidaemia is a major risk factor for developing CAD (Reiner *et al.*, 2011) as the disease aggregates atherogenic lipids which form plaques in interior artery walls (Lusis, 2000). Known as atherosclerosis, this is an inflammatory disease that targets large arteries in the body, however, it also affects arteries in the arms, legs or brain (Jacobson *et al.*, 2015). The potential to develop atherosclerosis is generated by genetic or environmental factors such as family history, gender, diabetes mellitus, high blood pressure, high LDLC levels or low HDLC levels, smoking or lack of exercise (Lusis, 2000). The exact cause of this type of CVD is unclear, but studies support the hypothesis that LDLC is a principal factor in inducing the development of atherosclerosis (Libby *et al.*, 2011). LDLC is an atherogenic lipoprotein, is used as a biomarker to define CVD risks and is therefore a target for the management of dyslipidaemia (Catapano *et al.*, 2016). Serum LDLC level of 4.13 mmol/L or more is defined as high, whereas a reduction of 1 mmol/L can reduce CVD mortality and morbidity by approximately 22% (Reiner *et al.*, 2011). In addition, a 10% reduction in HDLC levels increases the risk of CAD by 13% (Després *et al.*, 2000).

With respect to reducing mortality and morbidity related to hyperlipidaemia in individuals with low-moderate CVD risk, lifestyle improvements can be used as a preventive strategy to control elevated atherogenic lipid levels. Nutrition is an

important factor in reducing CVD risk as well as normalising lipid profile levels. For individuals with low-moderate hyperlipidaemia, the National Health Service recommends they adjust their lifestyle before considering hyperlipidaemic agents (NHS, 2018).

Statins treat hyperlipidaemia, and have been proven to potentially lower LDLC levels by 37% (Jacobson *et al.*, 2015), however, side effects have been recorded, including muscle and joint pain and an increased risk of developing diabetes (Sirvent *et al.*, 2012; Sattar *et al.*, 2014). In recent years, there has been an increasing interest in using nutritional supplements as an alternative to treating dyslipidaemia, e.g. PC. Previous studies (Childs *et al.*, 1981; Ovesen *et al.*, 1985; Polichetti *et al.*, 1998; Wat *et al.*, 2009; Mourad *et al.*, 2010; Lee *et al.*, 2014) have suggested that PC improves lipid profiles and decreases CVD risks by decreasing biomarkers associated with CVD and associated increasing HDLC levels. In contrast, some studies have not observed such effects in decreasing blood lipid levels (Oosthuizen *et al.*, 1998 and Kooshesh *et al.*, 2010). The diverging literature data may be due to differences in study design and PC intervention doses. PC dosages used in the literature vary between 500 mg (Mourad *et al.*, 2010) and 10.4 g (Childs *et al.*, 1981). Currently, there is no recommended daily intake for PCs, which makes it difficult to ascertain the precise beneficial dose for treating dyslipidaemia.

In light of the above, the main aim of this particular study was to examine the effects of two different PC doses (600 mg and 2400 mg), as a supplement, in individuals with mild elevated LDLC levels. Additionally, the literature provides limited data on the half-life effects of PC on lipid profiles. Thus, in this study, we determined the washout period for PC effects on lipid profiles over a four week period.

4.2 Methodology

4.2.1 Study participants and baseline characteristics

After obtaining ethical approval from UREC (Appendix 1b), study advertisements were posted on the OBU campus, public places in Headington (Oxford), such as supermarkets and cafés, and in the Oxford Daily Info news-sheet under the volunteering section. Advertisements were also posted at local medical centres around Oxford, after obtaining additional ethical approval (Appendix 1c).

The inclusion criteria were: adults aged between 18 to 65 years, LDLC level > 2.58 mmol/L and < 4.11 mmol/L and a BMI between 18 and 34.9 kg/m^2 (Appendix 1d). This age range was chosen because previous studies found that aging impaired hepatic lipid metabolism, resulting in increased lipid levels (Petersen *et al.*, 2003). Obese participants (class 2 and 3) were excluded as obesity affects lipid metabolism (Klop *et al.*, 2013) due to hormonal increases of leptin and adiponectin (Singla *et al.*, 2010). Recruitment of participants was based on their LDLC levels. This biomarker is used to define CVD risks in clinical research and practice and is therapeutically targeted in the management of dyslipidaemia (Catapano *et al.*, 2016). Levels were chosen above the desirable level and borderline high levels of LDLC based on National Lipid Association (NLA) classification of cholesterol and LDLC levels (Jacobson *et al.*, 2015). These levels were chosen based on NLA recommendation (2015) that hyperlipidaemic patients with LDLC between these levels must be advised first to adjust their lifestyle before starting a hypolipidaemic agent (Jacobson *et al.*, 2015).

Participants fulfilling the following criteria were excluded: 1) LDLC levels < 2.58 mmol/L or > 4.11 mmol/L, 2) BMI ≤ 17.9 or $\geq 35 \text{ kg/m}^2$, 3) pregnant and lactating women, 4)

smokers, 5) participants with renal disease, liver disease, diabetic mellitus, heart disease and hypo/hyperthyroidism, 6) usage of hypolipidemic agents, oral contraceptives, antihypertensive agents, anabolic steroids and diuretics, 7) usage of dietary supplements containing PC. Previously mentioned diseases and drugs were within the exclusion criteria as it shows that can affect LDL and/or TAGs levels (Jacobson *et al.*, 2015).

Participants were recruited from the 1st April 2016 until the end of September 2017. After recruitment, participants were randomly assigned using a randomisation program from the Psychology Department of Oxford Brookes University (<http://psych.brookes.ac.uk/research/order.asp>), to either of the two intervention groups. Before study commencement, the procedure was explained to all participants and they were provided with written copies, (Appendix 2b), after which they signed the consent form (Appendix 3b).

4.2.2 Study design

This was a parallel study design with two intervention groups, where participants were placed at random. The study duration was divided into two phases: an intervention phase and a washout phase. For the first four weeks, the first group (LDG) received a low PC oral supplementation dose (600 mg = one capsule), while the other group (HDG) received a high PC oral supplementation dose (2400 mg = four capsules). After completing the intervention period, participants continued to visit the nutrition centre every week for another four weeks to determine the PC washout period.

As previously stated, there is no precise recommended dietary PC intake as previous studies could not detect the exact effect dose of PC, therefore in this study the aim was to investigate if PC supplementation efficacy on lipid profile can be differentiate

from low dose to high dose. The doses used in this study were chosen based on manufacturer's recommendations based on the content of each capsule. Although up to 18 g of PC is considered safe (Kidd, 2002) the study protocol followed manufacturers recommendations by not exceeding four capsules per day.

During the first visit, participants completed a health questionnaire that included questions about disease, medication, supplements and smoking (Appendix 4b). Also, physical activity patterns were examined using the short version of the IPAQ (Appendix 6b; Craig *et al.*, 2003). Additionally, anthropometric measures were taken using a bio-electrical impedance analyser (Tanita BC-418 MA, Illinois, USA) to measure weight, body fat composition and BMI. Height was recorded using a stadiometer (Seca Ltd, Birmingham, UK). Participants were asked to wear light cloths on test days and measurements were taken without shoes.

4.2.3 Dietary measurements

Participants completed a dietary record to determine dietary habits and estimate the dietary intake of fats and cholesterol. The dietary record consisted of four sequential days (three week days and one weekend day, Appendix 9). A previously validated self-administrated FFQ (Chapter 2) was also completed in the first visit, to estimate daily dietary PC intake (Appendix 8).

4.2.4 Blood collection and measurement

At weeks 0, 2, 4, 5, 6, 7 and 8, participant's lipid profiles were measured. Lipid profiles were measured via finger prick (40 µl blood) by using the CardioCheck® blood analyser (Polymer Technology Systems, Inc. Indianapolis, USA), after 12 hours fasting. Capillary blood samples were collected by following WHO (2010) guidelines for capillary sampling procedures, using 1.8 mm lancets (Accu-Chek Safe-T-Pro Plus

Lancets, Roche Holding AG, Basel, Switzerland). CardioChek is a portable analyser that rapidly measures lipid profiles in whole blood samples (Whitehead *et al.*, 2014). TC, HDLC and TAGs lipid profiles were measured directly. LDLC was automatically calculated by the Friedewald formula: $LDLC = TC - HDLC - TAGs/5$ in mg/dl (Friedewald *et al.*, 1972).

4.2.5 PC supplementation

The study supplement used was PhosChol® polyenylphosphatidylcholine (PPC; Figure 4.1). It is 100% PC. The term PPC defined as a highly purified extraction obtained from soybeans with a standardised 3-*sn*-PC content of 72% - 96% (Gundermann *et al.*, 2016). The product was prepared by Nutrasal, Germany. The fatty acids composition of PPC is presented in Table 4.1 and the nutritional analysis of PhosChol® is shown in Table 4.2.



Figure 4.1 PhosChol® polyenylphosphatidylcholine (PPC) supplementation used by the study participants

Table 4.1 Fatty acids composition (%) of the polyenylphosphatidylcholine molecule from soybean*

Fatty acids	Percentage
Dilinoleoyl phosphatidylcholine DLPC (18:2-18:2)	40 – 52%
Palmitoyl-linoleoyl phosphatidylcholine PLPC (16:0-18:2)	23 – 24%
Oleoyl-linoleoyl phosphatidylcholine OLPC (18:1-18:2)	12 – 13%
Linoleoyl-linoleoyl phosphatidylcholine LLPC (18:3-18:2)	6 - 7%
Stearoyl-arachidonoyl phosphatidylcholine SLPC (18:0 – 18:2)	6%
Palmitoyl-oleoyl phosphatidylcholine POPC (16:0-18:1)	3 - 4%
Stearoyl-arachidonoyl phosphatidylcholine SAPC(18:0-20:4)	1 – 2 %

* Data is taken from the product information sheet provided by the manufacturer (Nutrasal, Germany).

Table 4.2 Nutrition information of the PC supplement (PhosChol)^a

Typical values	Four capsules	100 g
Energy	20 kcal	710 kcal
Total Carbohydrate	0.21 g	8.80 g
Fibre	0	0
Total fat	2 g	70.66 g
Saturated fat	0 g	5.6 g
Polyunsaturated fat	0.5 g	16.1 g
Monounsaturated fat	1.5 g	48.9 g
Phosphatidylcholine (as Polyenylphosphatidylcholine) from soy	2400 mg	
Cholesterol	n/a	<0.1
Sodium	n/a	19 ppm
Calcium	n/a	< 5 ppm
Potassium	n/a	0.07%
Iron	n/a	< 5 ppm
Phosphorus	n/a	1.2%
Thiamin	n/a	< 0.04 mg
Riboflavin	n/a	< 0.04 mg
Niacin	n/a	< 0.2 mg
Vitamin A	n/a	< 50 IU
Vitamin C	n/a	< 0.5 mg
Folic Acid	n/a	0.0887 mg/lb
Vitamin B12	n/a	< 0.002 mg/lb
Vitamin D	n/a	< 30.0 IU
Vitamin E	n/a	796 IU/lb

^a Data is taken from the product prescribing information sheet provided by the manufacturer (Nutrasal, Germany).

Kcal, Kilo Calorie; g, gram; ppm, part per million; mg/lb, milligram per pound; IU, international units; n/a, not applicable.

PC supplementation was supplied to the participant to be taken at home. The supplement was given to the participant at the first visit to cover two weeks' period and in the next visit, they were given the same amount to cover the last two weeks. At every visit, participants were asked to bring the capsule bottle to check compliance of the supplement intake. Each capsule contained 600 mg (LDG) of pure PC and four capsules contained 2400 mg (HDG). PC supplementation has not been recorded as causing any major side effects (Sirtori *et al.*, 1985); however, participants were advised if any adverse reactions were noted, they had to call the researchers. If more than transient mild gastrointestinal upsets or other illnesses persisted, participants would be advised to withdraw from the study.

4.2.6 Outcome measures

The primary outcome measure was LDLC levels after four weeks of PC supplementation. The secondary outcome measures were: TC, HDLC, TAGs, non-HDL-C and TC/HDLC ratios.

4.2.7 Statistical analysis

Sample size calculations were initially estimated on power ($1-\beta$ err prob) 80% according to a similar study (Høie *et al.*, 2005) where 54 participants were recruited, with 27 in each group. Calculations were demonstrated based on effect size of 0.8 of mean of change in LDLC and SD of 0.52mmol/L and α error type I of 0.05; however, difficulties were encountered in finding eligible participants for this study. Only 12 participants (n=7 in LDG and n=5 in HDG) completed the intervention phase and 10 participants (n=6 in LDG and n=4 in HDG) completed the washout phase.

A statistical power was calculated, using G*power (Version 3.1.9.2, Universitat Kiel, Germany), based on t-tests (two-tailed) and an effect size of 0.7 and β/α ratio = 1. This showed that the study power was 62%.

The Statistical Packages for the Social Sciences (SPSS) software, version 25.0, was used for statistical analysis. Normality of the data was determined using the Shapiro-Wilk test. Independent-sample t-tests were used to compare mean HDLC, Non-HDL-C and TC/HDLC ratios between the LDG and HDG groups. The TAGs, LDLC and TC values were not normally distributed, therefore the Mann-Whitney test was used to compare the median differences between the two groups. To assess changes over the four week period within each group, a one-way repeated measure ANOVA test was used to compare TC, HDLC, LDLC, Non-HDL-C and TC/HDLC ratios. The Friedman non-parametric test was used for TAGs, LDLC and TC values. A *P*-value less than 0.05 was recorded as statistically significant.

4.3 Results

4.3.1 Baseline characteristics of participants

Sixty-two participants were screened for LDLC levels between 2.59 to 4.11 mmol/L, of which 16 participants were initially eligible to take part. Of these, two were excluded: one due to a BMI higher than 40kg/m², while the other participant was taking beta-blockers which increase blood TAG levels (Jacobson *et al.*, 2015). One participant from the LDG phase withdrew after the first visit for personal reasons and one participant from the HDG phase was excluded due to PC dose non-compliance (Figure 4.2).

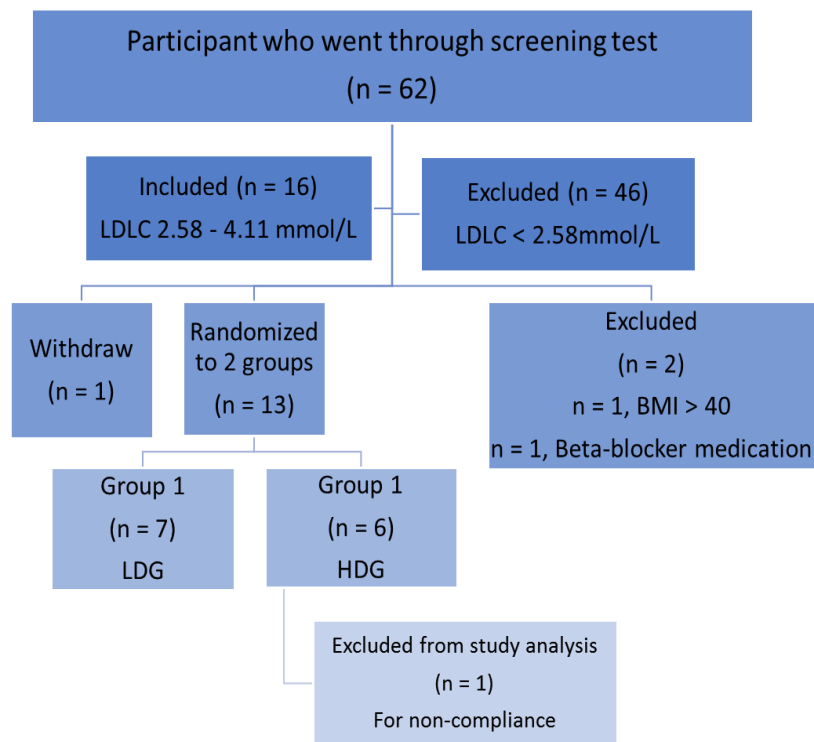


Figure 4.2 Flow diagram showing participant inclusion and exclusion

Participants were advised to contact the researcher in case of adverse effects. Overall, PC supplementation was well-tolerated; only one participant reported nauseous side-effects during the first three days. This participant was duly monitored and continued supplementation during the study period.

Table 4.3 summarises the baseline characteristics from the twelve participants. There were no significant differences in characteristics or in baseline lipid profile concentrations between the two groups. In addition, there were no significant differences in dietary fat intake.

Table 4.3 Baseline characteristics of study participants (n = 12)

Variables	LDG (600mg) n = 7	HDG (2400mg) n = 5	P value
Age (years)	49 ± 11	36 ± 11	0.161
Gender			
<i>Male</i>	3	3	
<i>Female</i>	4	2	
Height (cm)	166.4 ± 7.9	169.0 ± 6.6	0.750
Weight (kg)	70.0 ± 12.7	73.6 ± 9.6	0.700
Body fat percentage (%)	27.0 ± 8.2	25.9 ± 9.6	0.885
Body mass index (kg/m ²)	25.3 ± 4.6	25.8 ± 2.7	0.891
IPAQ score	1428.0 ± 1141.4	3831.6 ± 2040.4	0.100
Blood measurements			
TC (mmol/L)	5.6 ± 0.5	5.4 ± 0.5	0.777
TAGs (mmol/L)	1.0 ± 0.2	1.7 ± 0.8	0.353
HDLC (mmol/L)	1.6 ± 0.4	1.4 ± 0.2	0.349
LDLC (mmol/L)	3.2 ± 0.2	3.3 ± 0.6	0.817
Non-HDL-C (mmol/L)	3.6 ± 0.3	4.0 ± 0.7	0.313
TC/HDLC ratio (mmol/L)	3.3 ± 0.6	3.9 ± 0.7	0.297
Dietary measurements			
Cholesterol intake (mg/d)	278.7 ± 166.2	317.3 ± 198.0	0.737
Saturated fat intake (g/d)	29.0 ± 8.4	26.0 ± 4.7	0.788
Phosphatidylcholine intake (mg/d)	175.9 ± 64.5	267.8 ± 271.2	0.438

4.3.2 Intervention phase

The effects of PC supplementation on mean lipid profiles in the LDG and HDG groups are detailed in Table 4.4. Changes in lipids and lipoproteins were observed in both groups, however, none of these changes were significant. Both PC doses had a lowering effect on LDLC levels after four weeks. For TC and TAGs levels, both were decreased in the HDG group. The effect of increasing of HDLC was noticed in the LDG along with decrease in both groups for non-HDL-C. TC/HDL ratio levels were decreased in both groups, however, this was not significant.

Table 4.4 Effect of PC supplementation on lipid profile in the two groups*

Variables	LDG n = 7	HDG n = 5
Total cholesterol (mmol/L)		
Baseline	5.3±0.5	5.4±0.5
2 weeks	5.1±0.8	4.9±0.3
4 weeks	5.3±0.6	4.9±0.2
<i>P value</i>	<i>0.573</i>	<i>0.074</i>
TAGs (mmol/L)		
Baseline	1.0±0.2	1.6±0.8
2 weeks	1.2±0.3	1.6±1.0
4 weeks	1.2±0.7	1.2±0.3
<i>P value</i>	<i>0.156</i>	<i>0.819</i>
HDLC (mmol/L)		
Baseline	1.6±0.4	1.4±0.2
2 weeks	1.8±0.5	1.4±0.2
4 weeks	1.8±0.4	1.3±0.2
<i>P value</i>	<i>0.279</i>	<i>0.474</i>
LDLC (mmol/L)		
Baseline	3.2±0.2	3.3±0.6
2 weeks	2.8±0.3	2.8±0.3
4 weeks	3.0±0.6	3.0±0.1
<i>P value</i>	<i>0.156</i>	<i>0.504</i>
Non- HDL-C (mmol/L)		
Baseline	3.6±0.3	4.0±0.6
2 weeks	3.3±0.4	3.5±0.4
4 weeks	3.5±0.5	3.5±0.3
<i>P value</i>	<i>0.118</i>	<i>0.161</i>
TC/HDL ratio (mmol/L)		
Baseline	3.3±0.6	3.9±0.7
2 weeks	3.0±0.7	3.5±0.6
4 weeks	3.0±0.8	3.6±0.4
<i>P value</i>	<i>0.089</i>	<i>0.318</i>

*Mean ± SD; *P* values represent the changes within each group. Repeated measure ANOVA analyses were used to compare changes in variables (HDLC, Non-HDL-C, TC/HDL ratio) within each group. The Friedman test was used to compare changes in variables (TC, TAGs, LDLC) within each groups.

To detect more changes in lipid profile values after four weeks of PC supplementation, a comparison between the two groups was conducted (Table 4.5).

Table 4.5 Changes in lipid profile levels between the two groups, after four weeks of PC supplementation

Variables	Low dose n = 7	High dose n = 5	P Value
Total cholesterol (mmol/L)			
2 weeks	-0.1±0.6	-0.5±0.7	0.333
4 weeks	0.1±0.3	-0.5±0.3	0.007*
TAGs (mmol/L)			
2 weeks	0.2±0.3	0.0±0.8	0.629
4 weeks	0.2±0.6	-0.4±0.9	0.432
HDLC (mmol/L)			
2 weeks	0.2±0.4	0.0±0.1	0.429
4 weeks	0.2±0.2	-0.1±0.1	0.030*
LDLC (mmol/L)			
2 weeks	-0.4±0.3	-0.5±0.6	0.639
4 weeks	-0.2±0.5	-0.3±0.6	0.919
Non-HDL-C (mmol/L)			
2 weeks	-0.3±0.3	-0.6±0.8	0.493
4 weeks	-0.1±0.3	-0.5±0.4	0.128
TC/HDL ratio (mmol/L)			
2 weeks	-0.3±0.3	-0.4±0.8	0.779
4 weeks	-0.3±0.4	-0.3±0.6	0.943

Mean ± SD; **P* < 0.05 from baseline. Independent sample t-tests were used to compare changes in variables (HDLC, Non-HDL-C, TC/HDL ratio) between groups. The Mann-Whitney test was used to compare changes in variables (TC, TAGs, LDLC) between groups.

Total cholesterol levels were significantly different after four weeks of treatment, along with a significant difference in HDLC levels; however, there was a reduction in non-HDL-C in both groups. In both groups, there was a trend in LDLC level reduction. For TAGs and TC/HDL ratio levels, there were no significant differences between the two groups, but there were no changes in the LDG group, while there was a decreasing trend in the HDG group.

4.3.3 Washout phase

After four weeks of intervention, the elimination effects of PC supplementation on lipid profiles were measured after another four week period. Table 4.6 presents the changes in lipid profile after the discontinuation of the PC supplement in the two

groups. There were no significant changes from week one of discontinuing to week four; however, there was a decreasing trend in HDLC, in both groups.

Table 4.6 Lipid profile changes in the groups, during the four week washout period

Variables	LDG n = 6	HDG n = 4
Total cholesterol (mmol/L)		
Week 0	5.3±0.6	4.8±0.2
Week 1	5.3±1.1	4.8±0.1
Week 2	5.2±1.1	4.9±0.1
Week 3	5.0±0.7	4.7±0.2
Week 4	5.2±0.7	4.8±0.2
P value	0.650	0.590
TAGs (mmol/L)		
Week 0	1.2±0.7	1.2±0.4
Week 1	0.9±0.1	1.0±0.2
Week 2	1.4±0.8	1.3±0.4
Week 3	1.2±0.8	1.5±0.1
Week 4	1.3±0.2	1.5±1.0
P value	0.139	0.615
HDLC (mmol/L)		
Week 0	1.8±0.4	1.3±0.2
Week 1	1.8±0.5	1.5±0.3
Week 2	1.8±0.5	1.2±0.1
Week 3	1.7±0.5	1.2±0.1
Week 4	1.7±0.3	1.3±0.1
P value	0.760	0.424
LDLC (mmol/L)		
Week 0	2.9±0.6	3.0±0.1
Week 1	3.1±0.6	2.9±0.3
Week 2	2.8±0.8	3.1±0.3
Week 3	2.7±0.5	2.3±0.2
Week 4	2.9±0.5	2.8±0.2
P value	0.547	0.339
Non- HDL-C (mmol/L)		
Week 0	3.5±0.5	3.5±0.3
Week 1	3.5±0.7	3.4±0.3
Week 2	3.5±0.8	3.6±0.9
Week 3	3.3±0.5	3.5±0.2
Week 4	3.5±0.5	3.5±0.2
P value	0.833	0.792
TC/HDLC ratio (mmol/L)		
Week 0	3.8±0.8	3.6±0.5
Week 1	3.1±0.6	3.5±0.8
Week 2	3.1±0.8	4.0±0.2
Week 3	3.1±0.8	3.9±0.2
Week 4	3.1±0.5	3.8±0.3
P value	0.969	0.521

*Mean ± SD; $P < 0.05$. Independent sample t-tests were used to compare changes in variables (HDLC, Non-HDL-C, TC/HDLC ratio) within each group. The Mann-Whitney test was used to compare changes in variables (TC, TAGs, LDL-C) within each group.

A comparison between the LDG and the HDG groups of lipid profile measurements for the washout period after four weeks of PC treatment is presented in Table 4.7.

After 4 weeks of PC washout, changes in lipid biomarkers between groups were not significant. However, TAG blood levels were higher in the HDG group when compared to the LDG group.

Table 4.7 Lipid profile changes between groups in the four week washout period*

Variables	LDG n = 6	HDG n = 4	P Value
Total cholesterol (mmol/L)			
2 weeks	-0.0±0.6	0.0±0.3	0.882
4 weeks	0.0±0.4	-0.0±0.3	0.975
TAGs (mmol/L)			
2 weeks	0.2±0.6	0.1±0.7	0.762
4 weeks	0.0±0.6	0.4±0.9	0.546
HDLC (mmol/L)			
2 weeks	-0.0±0.2	-0.1±0.1	0.352
4 weeks	-0.1±0.2	-0.1±0.1	0.984
LDLC (mmol/L)			
2 weeks	-0.1±0.7	0.1±0.3	0.649
4 weeks	-0.0±0.5	-0.2±0.3	0.558
Non-HDL-C (mmol/L)			
2 weeks	-0.0±0.5	0.1±0.3	0.591
4 weeks	0.0±0.3	0.0±0.2	0.979
TC/HDLC ratio (mmol/L)			
2 weeks	0.0±0.3	0.4±0.3	0.103
4 weeks	0.0±0.5	0.2±0.2	1.000

*Mean ± SD; $P < 0.05$. Repeated measure ANOVA analyses were used to compare changes in variables (HDLC, Non-HDL-C, TC/HDL ratio) between groups. The Friedman test was used to compare changes in variables (TC, TAGs, LDLC) between groups.

4.4 Discussion

PC plays an important role in lipoprotein metabolism (Castro-Gómez *et al.*, 2015). Abnormalities during lipoprotein metabolism, including high levels of TC, LDLC or TAGs, or low levels of serum HDLC are defined as dyslipidaemia (Reiner *et al.*, 2011). Among the many factors that increase CVD risk, dyslipidaemia is highly relevant as the association between dyslipidaemia and CVD is clearly established in the literature (Nelson, 2013). Previous studies have demonstrated that PC supplementation can

significantly lower atherogenic lipid profile values and increase cardio-protective HDLC levels (Spilburg *et al.*, 2003; Høie *et al.*, 2005). However, the studies that examined PC supplementation on lipid profiles, showed significant beneficial effects on TC and LDLC using soybean lecithin; a PC product mixed with different components and PL ratios (Tompkins and Parkin, 1980; Medic` *et al.*, 2003). Thus, in this study we used a pure PC supplement to avoid any associated nutritional conflicts with other types of PLs. The precise dose of PC necessary to produce clinically significant improvements in the lipid profile has not been established. Therefore, the novelty of this study was the comparison of two different PC doses (600 mg, 2400 mg) on lipid profiles in individuals with mild elevated lipidemia. In addition, we assessed the effects of a washout period after PC supplementation on lipid profiles over a four week period, a study which has not been performed. The main finding was a significant reduction in TC levels ($P = 0.007$) when 2400 mg PC was administered for four weeks, when compared to the 600 mg dose. On the other hand, 600 mg of PC significantly increased HDLC levels ($P = 0.03$) after four weeks, when compared to the 2400 mg dose.

In this study, a significant reduction in TC levels was recorded when 2400 mg PC was administered to participants, when comparing the LDG and HDG groups; however, this was not significant within each group. This data agreed with a previous study where ~2 g PC was administered to hyperlipidaemic adults, showing a significant reduction in serum TC levels (Spilburg *et al.*, 2003; Høie *et al.*, 2005). In animal studies, dietary PC has been shown to stimulate biliary lipids. LeBlanc *et al.* (2003) investigated the effects of adding soybean PC to a rat diet, and found it stimulated bile flow and bile acid secretion, which was significantly associated with hepatic cholesterol secretion favouring a greater reversion of cholesterol to bile. In CVD, biliary cholesterol secretion

is important as it is the final step of the reverse cholesterol pathway (Wang and Rader, 2007). Additionally, dietary PC significantly reduced ACAT activity. This enzyme plays an importance role in promoting atherosclerosis and foam cell formation, by accumulating cholesterol esters in vascular tissue and macrophages (Katsuren *et al.*, 2001). Moreover, egg PC has been demonstrated to decrease the absorption of cholesterol in rats (Jiang *et al.*, 2001). It was questionable if PC sources, either from egg yolk or soybean, can change lipid metabolism; however, in one particular animal study, hydrogenated PC, either from egg or soy (0% PUFA), was fed to mice to investigate the effects on TC and TAGs hepatic lipids (Tandy *et al.*, 2010). After three weeks, both PC sources significantly reduced total liver lipid levels, when compared to control animals (Tandy *et al.*, 2010). Along with this effect, increases in hepatic cholesterol synthesizing enzymes and the LDL receptor were observed. Furthermore, the hepatic expression of cholesterol transporters was reduced. This effect of low levels of hepatic cholesterol may have arisen thanks to the lowered reflux of cholesterol from the intestine to the liver (Tandy *et al.*, 2010).

The reduction of the atherogenic lipoprotein, LDLC was a main aim of this study, as it plays a critical role in CVD pathogenesis (Catapano *et al.*, 2016). In this study, we observed a trending decrease (non-significant) in LDLC levels in response to PC in both groups. In a study conducted by Mourad *et al.* (2010), these authors found a significant reduction in LDLC level (56.11%) after eight weeks treatment of 500 mg soybean lecithin containing 340 mg PC, in twenty hyperlipidaemic patients. Similar results were also found in other studies (Spilburg *et al.*, 2003; Høie *et al.*, 2005). An *in-vitro* study by Desreumaux *et al.* (1979) examined the effects of different PLs on the activity of LPL and HTGL enzymes from rat heart, lung, liver and adipose tissues and healthy human

adipose tissue. Data suggested that PPCs significantly ($P < 0.001$) stimulated the activity of LPL and HTGL enzymes in these tissues. LPL and HTGL activation had generally been accepted to play roles in reducing LDLC and VLDL levels by breaking down lipoprotein dense TAGs (VLDL and IDL; Küllenberg *et al.*, 2012).

In relation to this interventional study, the short time duration and small sample size may have been an issue in terms of statistical power in detecting a statistically significant reduction in LDLC. It is worth noting that significant changes in TC levels recorded in our study, may have been due to appreciable changes in LDLC levels, considering that HDL particles have less effects on TC, when compared to LDLC and its smaller size (Childs *et al.*, 1981).

Recent guidelines have recommended the use of non-HDL-C as a primary target for CVD, as it is a better risk indicator than LDLC (NICE, 2014). Non-HDL-C is a biomarker that measures total atherogenic TAG-rich lipoproteins in blood (VLDL, VLDL remnants, IDL, Lp(a) and Apo B; Catapano *et al.*, 2016). Non-HDL-C is calculated by the formula: Non-HDL Cholesterol = Total Cholesterol – HDL cholesterol. In this study, no significant difference was found in non-HDL-C after four weeks of supplementation.

An important PC function is VLDL synthesis in the liver. Studies have shown that a choline deficient diet deteriorates PC synthesis, thereby accumulating TAGs in the liver and lowering plasma TAG levels due to impaired VLDL synthesis (Yao and Vance, 1988; Buang *et al.*, 2005). Moreover, in a study conducted by Beil and Grundy (1980), exogenous lecithin absorption mechanisms were investigated. It was found that lecithin infusion increased VLDL-TAG levels suggesting that lecithin enhances the formation of intestinal chylomicrons. In the current study, there were no significant changes in TAG levels in both groups, although in the LDG group, a slight increase in

TAGs was observed. TAGs levels were measured in whole blood samples in this study; a minor increase in TAG levels could have been due to VLDL particles, whereas in fasting participant samples TAGs were predominantly carried in VLDLs and their remnants (Chapman *et al.*, 2011). The role of VLDL is to carry TAG particles from the liver to the blood stream going to the peripheral tissues for storage in adipose tissue to avoid the accumulation of fat in the liver which causes fatty liver disease (Kwiterovich, 2000).

Furthermore, comparisons between the LDG and HDG groups showed statistically significant increases in HDLC levels ($P = 0.030$) for the LDG group. This observation agreed with previous studies which suggested that PC supplementation can significantly increase HDLC levels in dyslipidaemic patients (Childs *et al.*, 1981; Medic *et al.*, 2003). In a study by Zierenberg and Grundy (1982), the metabolic fate of dietary PC in men was studied. The authors showed that dietary PC rapidly fused into plasma HDL particles, after ingestion. The appearance of high PC levels in plasma HDL was accounted for by the direct secretion of PC into nascent HDL from enterocytes (Blesso, 2015). The significance of PC in HDL has been shown to be a major factor in its ability to accept cholesterol from peripheral cells during reverse cholesterol transport (Yancey *et al.*, 2000). In 1998, Polichetti and colleagues examined the effects of oral PC supplementation on HDL metabolism. They found that the administration of pure PC (3.04 g) to ten hypercholesterolemia patients over six weeks, significantly increased Apo AI-HDL lipoprotein levels (Polichetti *et al.*, 1998). The main protein component of HDLC is Apo AI. The regulation of Apo AI impacts on HDLC levels as it is responsible for LCAT activation (Lewis and Rader, 2005). This process supports the transformation of

reverse cholesterol by converting free cholesterol to HDLC, by hydrolysing cellular cholesterol esters (Küllenberg *et al.*, 2012).

A strong inverse relationship between high HDLC levels and CVD risk has been reported in the literature (Gordon and Rifkind, 1989). Interventional studies have suggested that a 1% increase in HDLC can lead to a 3% reduction in CVD risk (Manninen *et al.*, 1988). Currently, statins and fibrates increase HDLC levels by 10% and similarly niacins work by improving HDLC levels by up to 25% (Catapano *et al.*, 2016). Combining these drugs with PC supplements may help increase HDLC levels.

The second aim of this study was to assess the elimination effects of the PC supplement on lipid profiles, over a four week washout period after the intervention phase. Washout period defines the period of time during a clinical study (cross-over studies) when a participant stops taking a study drug in order to eliminate the effects of the treatment (Roberts *et al.*, 2015). This may also refer to a withdrawal from a current treatment before an active treatment begins (Roberts *et al.*, 2015). The importance of the washout period in research is to reinforce the carry-over effect of the current treatment to the second active treatment. In addition, the washout period can help to identify the long-term adverse effect of the drug after discontinuing it (Roberts *et al.*, 2015).

It was indicated in an early study that total PC absorption is approximately 26% and 50% after 9.5 h and 19 h, respectively (Lekim, 1976). Currently, there is a lack of data on how long the impact of oral PC supplementation on lipid profile can last after the experimental phase. The data from this study did not show any significant results in terms of increasing atherogenic lipoprotein after halting PC supplementation.

Although there was a significant reduction in TCs and a significant increase in HDLC, four weeks of washout could not detect any systematic changes in lipid biomarkers.

It was recorded previously that the washout period of a drug is equal to five elimination half-lives (Trepanier, 2013). The elimination half-life defines as the period required for the volume of drug in the body to reduce by half (Nnane, 2005). In a previous study (Nicolson and Ash, 2014), it was reported that the half-life of high concentrations of injected intravenously PLs into the circulation was rapidly decreased by 80% within 15 min of injection. Moreover, residual PLs slowly declined with a half-life of 59 days (Wagener, 1972). However, this may not be a good model for dietary or oral PLs turnover in the blood circulation as the concentrations of PLs in the blood are many times higher after intravenous injection than those transported from intestinal sources (Wagener, 1972). In the current study, four weeks was chosen as was considered the necessary washout period for patients under treatment with antihyperlipidemic agents (Yokoyama *et al.*, 2003). A longer duration of washout period with a larger sample size may be more likely to find the exact elimination effect of oral PC supplementation on lipid profile.

From the literature, the efficacy of oral PC supplementation on improving lipid profile levels are inconsistent. Whereas some studies found improvements (Childs *et al.*, 1981; Spilburg *et al.*, 2003; Høie *et al.*, 2005) others did not detect any changes in lipid profiles (Kesaniemi and Grundy, 1986; Oosthuizen *et al.*, 1998). Several factors could be behind these inconsistencies. One involves the method of oral PC administration. The consumption of PCs with food is more likely to trigger the secretion of biliary lipids that promote intestinal absorption (Burgees *et al.*, 2004). This factor was considered in this study and participants were instructed to take the supplement with meals.

Secondly, PCs doses varied across studies; e.g. 10.4 g (Childs *et al.*, 1981), 3.04 g (Polichetti *et al.*, 1998), 550 mg (Spilburg *et al.*, 2003) and 500 mg (Mourad *et al.*, 2010). In this study, two doses were used; 600 mg represent the control group and 2400 mg was the recommended dosage from the manufacture. The high dose used in this study considered as an average dose compared to the previous used doses in the literature. As there is lack of DRVs for PC, it is difficult to decide a correct dose to treat hyperlipidaemia. The study duration also varied in previous studies, as it ranged between four and 24 weeks (Buchman *et al.*, 1992; Medić *et al.*, 2003).

In this study, the intervention phase was four weeks as it is the minimum recommended period in which to record changes in lipid profiles when a new hypolipidaemic agent is administered to hyperlipidaemic patients (Safeer and LaCivita, 2000). In addition, the four weeks period was thought to minimize the drop-out rate and was considered ideal given the time constraint for this project. Though significant changes were recorded for TC and HDLC levels, a longer intervention period may help to detect significant changes in LDLC levels. Moreover, dietary and lifestyle changes can affect lipid profiles, including increased alcohol consumption or increased dietary fat intake or even increased physical activity. For this reason, in this study participants were encouraged to maintain their regular diet and physical activity over the eight weeks to limit potential lipid improvement cofactors. However, no specific low-fat low-cholesterol diet was imparted to participants as in other studies which combined PC supplementation with a low-fat low-cholesterol diet (Spilburg *et al.*, 2003).

4.5 Limitations and conclusions

This study examined the efficacy of two different PC doses among mild hyperlipidaemic individuals. It also aimed to determine the elimination effect of PC

supplementation on lipid profile through a washout period, over a four week period, after treatment. The main strength of this study was its design, as it excluded cofactors, such as smoking, drugs and disorders that can affect lipid profiles that some of them were not excluded in previous studies. In addition, a pure PC supplementation was used in this study to avoid any conflict of efficacy with other types of PLs.

This study showed that 2400 mg oral PC supplementation significantly decreased TC levels, whereas 600 mg significantly increased HDLC levels. However, the generalisation of these results is subject to some limitations, e.g. the small sample size. Though a study aim was to recruit 54 participants, with 27 in each group, finding eligible participants was difficult. Although, 62 participants were screened, only twelve were eligible and completed the study. If we could reach study power 80%, statistical significant changes in LDLC and non-HDL-C levels would perhaps be recorded. Also, study participants were not allocated to groups based on age or BMI stratification. This was difficult to perform due to the restricted project timelines.

Due to cost considerations, the accurate assessment of capsule compliance and causality of PC supplementation in improving lipid profile was difficult. In this study, capsule compliance was assessed through questioning participants and the return of unused capsules. Ideally we would have measured PC serum concentrations or serum linoleic acid levels before and after intervention to monitor compliance. In addition, as this study showed that PC supplementation significantly lowered serum TC levels and increased serum HDLC levels, clarification of the causality of PC supplementation in improving lipid profile is required. This could be done by measuring Apo-AI and Apo-B100 levels.

Another limitation of the study was the four week intervention period. If the study duration was longer (eight weeks) this may have allowed the detection of significant result within each group.

Although the current study is based on a small sample of participants, the findings suggest the potential use of PC supplementation to help dyslipidemic patients to reach their ideal goal of HDLC (> 1.0 mmol/L). However, further work is needed to support these results.

Chapter 5: General discussion and conclusions

Genetic and environmental factors underpin CVD pathogenesis (Reiner *et al.*, 2011). Hyperlipidaemia is a risk factor for the development of CVD where one-third of heart diseases are related to high levels of lipidaemia (WHO, n.d.c). In 2009, in the UK, mean TC levels for males and female >25 years were 5.4mmol/L, whereas normal TC levels are <5 mmol/L (WHO, n.d.b). Additionally, there has been an increase in prescriptions for lipid-lowering drugs as since the late 1990s these have increased six fold (Bhatnagar *et al.*, 2015). Many lipid-lowering drugs have been approved to treat hyperlipidaemia and ultimately lower CVDs. These drugs target lipid metabolism in the intestine by inhibiting the cholesterol absorption. Statins, which have been used to treat hyperlipidaemia, have proven to potentially lower LDLC and TAGs levels by 37% and 19%, respectively (Jacobson *et al.*, 2015). However, statin side-effects have been observed such as muscle and joint pain and an increased risk in developing diabetes (Sirvent *et al.*, 2012; Sattar *et al.*, 2014).

Nutraceutical supplements have been frequently proposed in terms of hyperlipidaemia management (Houston, 2014). One of these supplements is phospholipids, which play a vital role in the body as it is a cellular membrane component. Additionally, it is a major component of all lipoproteins (HDL, LDL, IDL, VLDL and chylomicrons), which contribute to fat transportation inside the body (Cohn *et al.*, 2010). PC is the most common phospholipids in foods; moreover PC is derived from the bile, approximately 12 g of PC daily. The importance of biliary PC lies in its essential role in the emulsification of dietary lipids (Hişmioğullari *et al.*, 2007).

Research to date has not yet fully elucidated the relationship between dietary PC and CVD pathogenesis. Where many studies have shown that PC supplementation is effective in normalising lipoprotein metabolism in hyperlipidaemic patients (Polichetti *et al.*, 1998; Mourad *et al.*, 2010), other studies have questioned whether the metabolism of dietary PC causes inverse effects and increases CVD risk by producing TMAO an atherogenic factor (Wang *et al.*, 2011; Tang *et al.*, 2013). Much less is known, however, about the role of dietary intake and supplementation of PC in normalizing lipoprotein levels among UK individuals. In light of these equivocal studies, we conducted three studies to help answer our research question: to which extent is the dietary intake and supplementation of PC related to cardio-metabolic biomarkers?

5.1 General discussion and findings

The primary aim of this thesis was to determine the effects of dietary PC on blood lipid biomarkers (TC, LDLC, HDLC and TAGs) in healthy and hyperlipidaemic adults, using either natural food sources or oral supplementation (Chapters 3 and 4). The secondary aim was to develop and validate a self-administered FFQ to measure dietary PC intake among the UK population (Chapter 2). To achieve these aims, three studies were conducted: i) a study to validate a newly developed FFQ to measure dietary PC intake in reference to a 5-day weighed-record (Chapter 2); ii) a cross-sectional study to assess the relationship between dietary PC intake and cardio-metabolic biomarkers (Chapter 3); and iii) an intervention study to test the efficacy of PC supplementation on lipid profiles among hyperlipidaemic individuals (Chapter 4).

To explore the relationship between PC intake and cardio-metabolic biomarkers, we developed a valid nutritional tool to measure dietary PC intake (Chapter 2). To the best of our knowledge, this is the first study to develop a valid self-administered FFQ to

measure dietary PC intake. In large epidemiological studies, FFQs are the most commonly used data gathering mechanism, thus in this particular study, a validated FFQ was necessary to address the relationship between dietary PC intake and cardio-metabolic biomarkers. Therefore, a 136-item self-administrated FFQ was validated in reference to a 5-day weighed record. The consumption of dietary PC was also measured in a select UK population using this validated FFQ. The findings suggested that the validated FFQ showed an acceptable agreement using the Bland-Altman plots. This was ascertained when differences in intake between the two methods (FFQ and WR) were plotted against the mean intake of the two measures, suggesting this FFQ was applicable to future research among UK populations. In addition, a minor adjustment of the validated FFQs by adding commonly consumed foods for the study population and assess its validity is recommended to be used in different populations. Moreover, the measurement of dietary PC consumption showed that the average intake of dietary PC among healthy individuals in the UK was 255 mg per day. However, there is no recommended daily intake for PC, therefore we cannot be certain if this level is sufficient for human health or not. This first study was an important basis for the second study (Chapter 3) to assess the correlation of dietary PC intake in relation to cardio-metabolic biomarkers in healthy populations.

The second study (Chapter 3) was a cross-sectional study addressing the association between dietary PC intake and cardio-metabolic biomarkers among healthy adults in the UK. Until now, there was a gap in the literature in terms of PC and associated nutritional aspects, including a database containing the PC content in common foods where the only one was released in 2008 (Patterson *et al.*, 2008). Moreover, data on

the association between dietary PC and blood lipid profiles and CVD is limited as only one previous US study has looked into this topic (Zheng *et al.*, 2016).

Thus, this study was not only timely but warranted in the UK, as it sought to measure dietary PC using a validated FFQ and examine potential associations with blood lipid profiles. The results showed a significant ($P = 0.017$) inverse relationship between dietary PC intake with blood TAG levels, thereby confirming the previous link between dietary PC and hepatic TAG synthesis (Buang *et al.*, 2005). As the new trending question is debating whether dietary PC metabolism can promote CVD, a correlation between dietary PC and Lp(a) was not found. However, a limitation of this result is that the majority of participants had normal Lp(a) levels. A future research to assess the association between dietary PC intake and Lp(a) levels among older participant with abnormal levels of Lp(a) is recommended.

The third study (Chapter 4) was a randomised study to investigate the efficacy of PC supplementation on lipoprotein levels among hyperlipidaemic individuals in the UK. Currently, there is no nutrient reference for PC intake in healthy adults, thus this study compared two different PC doses (600 mg and 2400 mg) to explore lipid biomarker differences with respect to low or high PC dose supplementation. The secondary aim of this study was to investigate the long-term effect of PC supplementation on lipid profile by assessing lipid profile over four weeks washout period. The results showed a significant reduction in TC levels for the high dose group and an increase in HDLC levels for low dose group. However, washout period results did not show any significant outcomes in terms of increasing lipid profile levels after withdraw PC supplementation. Based on the current findings, it is difficult to recommend between 600 mg or 2400 mg of PC dose due to the small study population. General findings of the study suggest the

potential of using PC supplementation as a therapeutic agent to normalize hyperlipidaemia. However, longer study duration and a bigger sample size is recommended to explore the efficacy of PC supplementation on lipid profile.

5.2 Study limitations, implications and applications

The main limitation of this work was the limited number of participants. Many factors restricted the recruitment of more participants, for example in study one (Chapter 2) and study two (Chapter 3), resource and time constraints restricted the recruitment of more participants. In study three (Chapter 4), the inclusion criteria and long study duration (eight weeks) made recruitment a challenge. Moreover, limited resources prevented assessment of TMAO level in study 2 (Chapter 2) and the causality in study 3 (Chapter 3) of the effect of oral PC supplementation on blood lipid biomarkers by measuring apolipoprotein markers.

In research related to dietary fat, challenges remain for a number of reasons. Firstly, dietary fat is one of the most difficult dietary components to assess through nutritional assessment methods as it is widely dispersed throughout the diet and sometimes it is very difficult for an individual to quantify the amounts that have been used for food preparation (for example, frying or as sauces and dressings). Moreover, the evaluation of the factors that influence the circulating apolipoprotein levels of individuals, including genetic variation, medical history status, lifestyle differences (for example, alcohol consumption and smoking), are all factors that are necessary to be considered in this area of research as it can affect the accuracy of the study outcomes.

In the UK, there are an estimated seven million people living with CVD (BHF, 2018). Epidemiological studies have demonstrated a strong relationship between high blood

cholesterol levels and TAG levels and an increased risk of atherosclerotic CVD (Nelson, 2013); however, less is known about the role of phospholipids in CVD pathogenesis. This thesis has raised the profile of dietary PC in diets and supplements and has increased the potential of PC as a nutraceutical to treat hyperlipidaemia. The translation of theory into practice is important in health related studies. The first objective of this project was to validate a self-administrated FFQ to measure dietary PC intake. This thesis recommends the utilisation of these FFQ in future epidemiological and interventional studies as a useful tool in measuring dietary PC intake, adapted to the specific study population. Further work to improve the FFQ validity involves the development of a PC-based food database and the updating of nutrition analysis software to include PC content is also recommended. The second objective of the study was to determine to what extent dietary and PC supplementation affected lipoproteins levels. Study 3 results provided further evidence that PC plays a role in lipid metabolism; however, further work assessing the association of long term dietary PC intake as an individual risk factor with CVD, using a bigger and more varied sample, is recommended. In addition, the outcome of Study 3 is promising as it suggests the potential of using PC supplementation as a hypolipidemic agent. PC supplementation can be an option for patients with adverse side effects from hypolipidemic drugs, or for patients with mild hyperlipidaemia, who must modify lifestyles. In addition, it is encouraging to explore the effects of PC supplementation in bigger sample sizes to confirm our study results.

5.3 Recommendations for future research

The results from this thesis suggest that dietary and oral PC supplementation is important for normal lipoprotein metabolism. However, a number of key research questions remain to be addressed and further research should be undertaken to:

- Fully and comprehensively validate the FFQ in a larger sample size and evaluate its reproducibility. Also, the PC content should be listed in common foods in the UK and in commonly used databases, e.g. the McCance and Widdowson database. Moreover, establishing a DRV for PCs in healthy UK adults would be beneficial in deciding correct PC doses and the precise levels crucial for normal lipid metabolism.
- Although the effects of dietary PC on health is subject to debate, it is important to study the influence of different PC fatty acids on lipid metabolism and CVD progress. Additionally it is vital to understand metabolic PC pathways and initiate *in vitro* studies of PC digestion co-factors. Thus, the effect of dietary PC on lipid profile and TMAO production to determine their combined impact on risk of CVD is recommended. Also, it is crucial to determine safety limits for the consumption of PC to avoid the reversible effects of dietary PC. As acute dietary PC effects on CVD development have been previously studied (Tang *et al.*, 2013); however, the effect of chronic consumption of dietary PC on CVD risk and an optimal dose for PC where the PC promoting aspects outweighs any possible TMAO effect on CVD risk remain to be explored.
- More studies are required to investigate oral PC intervention as a preventive treatment for hyperlipidaemia patients over longer periods (> four weeks). Moreover, a further study to explore the washout period for PC

supplementation effect on lipid profile over longer periods (> four weeks) is recommended. As a combination therapy of antihyperlipidemic agents is a common practice in dyslipidaemic patients, it is suggested that future studies explore the impact of adding PC supplementation to hypolipidaemic drugs (statins) on the overall hypolipidaemic effect. More understanding into the causality of PC effectiveness is important by exploring the effects of PC on lipoproteins fractions, for example, HDL2 and apolipoproteins (Apo B100 and Apo B48).

5.4 General conclusions

This thesis provides novel insights into dietary PC and how as a molecule it can be exploited in the fight against hyperlipidaemia and CVD. The results from this thesis contribute to our understanding of nutritional supplements like PC (lecithin) and how they impact on blood lipid profiles. The thesis promotes PC supplementation as a therapeutic agent to treat hyperlipidaemia and to establish treatment protocols both in the clinic and the home.

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Appendices

Appendix 1: Ethical approval

Appendix 2: Participant information sheet

Appendix 3: Consent form

Appendix 4: Basic health questionnaire

Appendix 5: Alcohol consumption assessment

Appendix 6: International physical activity questionnaire

Appendix 7: Weighed food record sheet

Appendix 8: Food frequency questionnaire

Appendix 9: Daily diet record

Appendix 1a

Dr Helen Lightowler
Director of Studies
Department of Sport and Health Sciences
Faculty of Health and Life Sciences
Oxford Brookes University
Headington Campus

17 November 2016

Dear Dr Lightowler

UREC Registration No: 161043

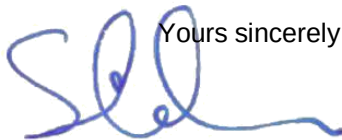
Dietary phosphatidylcholine assessed by food-frequency questionnaire in relation to lipid biomarkers in healthy populations

Thank you for your email of 26 October 2016 outlining your response to the points raised in my previous letter about the PhD study of your research student Majd Jan and attaching the revised documents. I am pleased to inform you that, on this basis, I have given Chair's Approval for the study to begin.

The UREC approval period for this study is two years from the date of this letter, so 17 November 2018. If you need the approval to be extended please do contact me nearer the time of expiry.

Should the recruitment, methodology or data storage change from your original plans, or should any study participants experience adverse physical, psychological, social, legal or economic effects from the research, please inform me with full details as soon as possible.

Yours sincerely



Dr Sarah Quinton
Chair of the University Research Ethics Committee

cc Sangeetha Thondre and Alaaddine El-Chab,
Supervisory Team Majd Jan, Research Student
Anne Delextrat, Research
Ethics Officer Jill Organ,
Research Degrees Team
Louise Wood, UREC
Administrator

Appendix 1b



Dr Catherine Kerr
Director of Studies
Department of Sport and Health Sciences
Faculty of Health and Life Sciences
Oxford Brookes University
Gipsy Lane
Headington

7 December 2015

Dear Dr Kerr

UREC Registration No: 150964
The effects of dietary lecithin on cholesterol and lipid profile

Thank you for the email of 26 November 2015 outlining your response to the points raised in my previous letter about the PhD study of your research student Majd Jan and attaching the revised documents. I am pleased to inform you that, on this basis, I have given Chair's Approval for the study to begin.

The UREC approval period for this study is two years from the date of this letter, so 7 December 2017. If you need the approval to be extended please do contact me nearer the time of expiry.

Should the recruitment, methodology or data storage change from your original plans, or should any study participants experience adverse physical, psychological, social, legal or economic effects from the research, please inform me with full details as soon as possible.

Yours sincerely

A handwritten signature in blue ink, appearing to read "S Quinton", with a long horizontal flourish extending to the right.

Dr Sarah Quinton
Chair of the University Research Ethics Committee

cc Helen Lightowler, Second Supervisor
Majd Jan, Research Student
Dido Green, Research Ethics Officer
Jill Organ, Research Degrees Team
Louise Wood, UREC Administrator

Appendix 1c

Dr Helen Lightowler
Director of Studies
Functional Food Centre
Department of Sport and Health Sciences
Faculty of Health and Life Sciences
Oxford Brookes University
Headington

18 August 2016

Dear Dr Lightowler

UREC Registration No: 150964
The effects of dietary lecithin on cholesterol and lipid profile

Thank you for your email of 17 August 2016 requesting a change to the original study approved by UREC on 7 December 2015 for your research student Majd Jan.

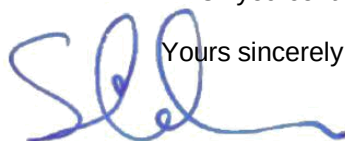
You wish to recruit participants by displaying the study advertisement in the waiting area of local medical centres. Advice has been sought and this method of recruitment does not require HRA R&D as GP practices are outside of this, providing all research activity including consent is undertaken off site.

A letter to the medical centres and a copy of the advertisement has been provided and on this basis I give Chair's approval for this change. The UREC approval remains the same as the original study, so until 7 December 2017.

Should the recruitment, methodology or data storage change from your original plans, or should any study participants experience adverse physical, psychological, social, legal or economic effects from the research, please inform me with full details as soon as possible.

I wish you continued success with your research.

Yours sincerely



Dr Sarah Quinton
Chair of the University Research Ethics Committee

cc Majd Jan, Research Student
Dido Green, Research
Ethics Officer Jill Organ,
Research Degrees Team
Louise Wood, UREC
Administrator

Appendix 1d

Majd Jan
PhD Student
Department of Sport and Health Sciences
Faculty of Health and Life Sciences
Oxford Brookes University
Headington Campus

7 June 2017

Dear Majd

UREC Registration No: 150964
The effects of dietary lecithin on cholesterol and lipid profile

Thank you for your email of 5 June 2017 requesting a minor amendment to the original study approved by UREC on 7 December 2015.

I confirm that you wish to extend the age range of potential participants from 18-60, to 18-70. You have provided a copy of the updated participant information sheet and study poster for the UREC file. On this basis I give Chair's approval for this change. The UREC approval remains the same as the original study, so until 7 December 2017.

Should the recruitment, methodology or data storage change from your original plans, or should any study participants experience adverse physical, psychological, social, legal or economic effects from the research, please inform me with full details as soon as possible.

I wish you continued success with your research.

Yours sincerely



Dr Sarah Quinton
Chair of the University Research Ethics Committee

cc Helen Lightowler,
Supervisory Team Anne
Delextrat, Research Ethics
Officer Jill Organ, Research
Degrees Team Louise Wood,
UREC Administrator

Appendix 2a

Dietary phosphatidylcholine assessed by food-frequency questionnaire in relation to lipid biomarkers in healthy populations

Participant Information Sheet

Please take your time to read the following information of this research study carefully and then decide whether or not you wish to take part. Thank you for reading this.

What is the purpose of this study?

We would like to measure the dietary intake of phosphatidylcholine (PC) among a healthy UK population and to find out if dietary intake of PC has an impact on lipid profile, for example total cholesterol. We also aim to assess the association between dietary intake of PC and the concentration of PC in blood.

Phosphatidylcholine is a type of fat, and can be found in different food sources such as egg yolk, liver, soybean and beef. PC is important for transportation and digestion of fat in our body.

In this study we plan to measure the daily intake of dietary PC in healthy individuals and to determine if there is any relation between intakes of PC and lipid profile. We wish to study about 100 adult volunteers (men and women) aged between 18 to 65 years old. **It will take 2 visits for each person to complete the study which takes around 1 hour.**

Why have I been invited? _

You are invited to be a participant in this study because you are medically free from diabetes, hypertension, renal, liver and heart diseases and your age is between 18 -65 years.

Do I have to take part?

You are being asked to participate in this study as a **volunteer**. If you decide to take part, you will be given this information sheet to keep and be asked to sign a consent form – but you will be free to withdraw at any time and without giving a reason. If you are a student or staff at Oxford Brookes University, your decision to take part or not will not affect your academic progress or the regard with which you are held in the University, now or in the future.

What are the inclusion and exclusion criteria?

Inclusion criteria:

- Adult (Men / woman) aged between 18 – 65 years old

Exclusion criteria:

- People with diabetes, hypertension, renal, liver diseases and heart diseases
- Pregnant

What will happen to me if I decide to take part in the study?

You are required to come for two visits to the Functional Food Centre at Oxford Brookes University.

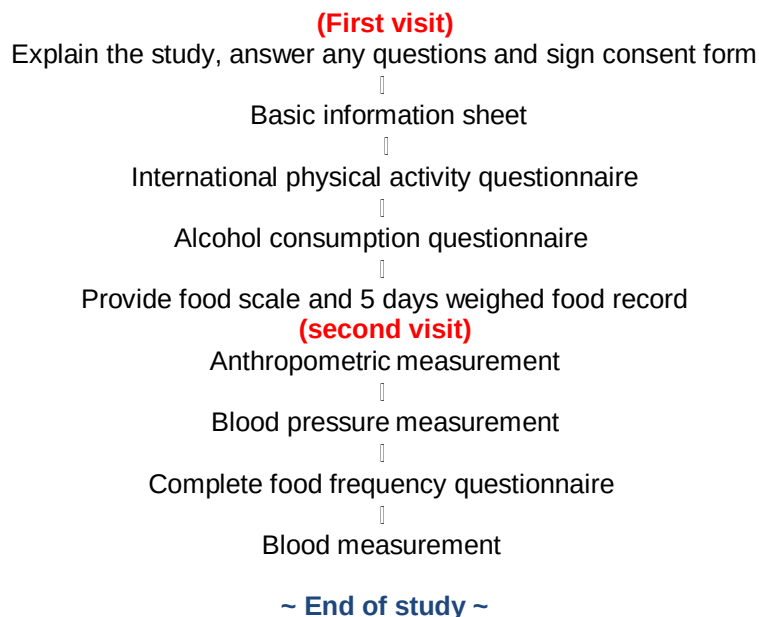
At your first visit, you will:

- Asked to sign consent form and then the researcher will record some basic information.
- Complete a physical activity chart to determine your activity level.
- Complete Alcohol consumption questionnaire.
- Be provided with a food weighing scale and food diary to fill out 5 days weighed food record.

The food scale is property of the Functional Food centre which means you should return it back to the researcher in your next visit.

At your second visit:

- Give the following anthropometric measurements including: weight, height, body fat percentage and waist and hip circumference.
- Be measured your blood pressure.
- Blood measurement will be taken by the **venepuncture method** to measure your lipid profile, PC level and lipoprotein(a) in your blood.
- Complete a food frequency questionnaire of phosphatidylcholine which include 136 items (this will be filled by the participant).



Are there any possible disadvantages or risk if I choose to take part?

There are limited risks in participating in this study. However we will need to take blood samples in your visit which may give some discomfort, but these will be taken by a trained researcher.

What are the possible benefits of taking part?

- You will be able to have your baseline anthropometric values and body composition.
- You will be sent, after analysis of the data, a report of your daily intake of PC and results of your lipid profile, on request.
- At the end of the study you will be given a £15 amazon voucher and a snack as a thank you for your time.

Will the study results be kept confidential?

Your right to privacy will be respected. All information collected about you during this study will be kept strictly confidential, subject to legal limitation. The information about you will be stored on a secure password protected database, only accessible by the research team. You will not be identified individually in any written or spoken presentation of the study. You will be allocated a study number which will be used as a code to identify you on the study database and kept securely for a period of 10 years after the study has finished, after then the information will be destroyed.

What will happen to the results of the research study?

The results will be presented as a part of a PhD thesis and in scientific poster or journals however, all data published will be de-identified.

Who is organising and funding the research?

The research is being conducted as part of PhD project under the supervision of Dr Helen Lightowler, Dr. P. Sangeetha Thondre and Dr. Alaaddine El-Chab at the Department of Sport and Health Sciences at Oxford Brookes University. This study is self-funded by the research student (Majd Jan).

Who has reviewed the study?

This research study has been reviewed and approved by the University Research Ethics Committee (UREC). If you have any concerns about how this study has been conducted you can contact the University Research Ethics Chair on ethics@brookes.ac.uk

Contact for further information

You can contact the researcher at any time if you have any questions or concerns

Ms Majd Jan

Email: 14097361@brookes.ac.uk

Dr Helen Lightowler

Email: hlightowler@brookes.ac.uk

Dr Sangeetha Thondre

Email: pthondre@brookes.ac.uk

Dr Alaaddine El-Chab

Email: a.el-chab@brookes.ac.uk

Thank you for your time to read this information sheet.

Participant Information Sheet

The effects of dietary lecithin on cholesterol and lipid profile

You are being invited to take part in a research study. Before you decide whether to do so, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and then decide whether or not you wish to take part. Thank you for reading this.

What is the purpose of this study?

We would like to study the effect of dietary lecithin on improving lipid profile particularly cholesterol and Low density lipoprotein (LDL)-cholesterol in individuals with mild hypercholesterolemia and to determine how long the effect of lecithin on the blood lipid profile may last.

Lecithin is the commercial name of phosphatidylcholine a class of the phospholipids (a type of fat). It can be found in different food sources such as egg yolk, liver, soybean and beef. The role of phosphatidylcholine in the body is important as it is a major part of all lipoproteins, which help in fat transportation inside the body as well as being one of the biliary lipids that help in fat digesting and metabolism.

In this study we plan to compare the effect of two different doses of lecithin taken for a total of four weeks and after that we will test participants over another four weeks to determine the exact washout period of the lecithin effect on the lipid profile. We wish to study about 30 adult volunteers (men and women) aged between 18 to 70 years old. It will take 8 weeks for each person to complete the study.

Why have I been invited? _

You are invited to be a participant in this study because you are an adult with above desirable or borderline high level of LDL-cholesterol.

If you are not sure about your cholesterol level but you are an overweight or you think you are following an unhealthy life style then you are welcome to come for a screening visit.

You have confirmed that you are non-smoker, with no history of any medical condition such as diabetes, hypo/hyperthyroidism, renal, liver and heart diseases. Finally you should not be pregnant and/or lactating.

We would like to check your LDL status. This will enable us to examine if lecithin intake would help you to improve your lipid profile.

Do I have to take part?

You are being asked to participate in this study as a **volunteer**. If you decide to take a part you will be given this information sheet to keep and be asked to sign a consent form – but you will be free to withdraw at any time and without giving a reason. If you are a student or staff at Oxford Brookes University, your decision to take part or not will not affect your academic progress or the regard to which you are held by the University, now or in the future.

What are the inclusion and exclusion criteria?

Inclusion criteria:

- Adult aged 18 – 70 years old.
- LDL level more than 2.58mmol/l and less than 4.11mmol/l (will be measured by the researcher)
- BMI level between 18 to 34.9kg/m² (will be measured by the researcher)

Exclusion criteria:

- A desirable level of LDL-cholesterol less than 2.58mmol/l or more than 4.11mmol/l
- BMI $\geq$ 35kg/m²
- Pregnant and/or lactating
- Smokers.
- Diseases requiring drug intervention such as renal disease, liver disease, diabetic, heart disease, hypo/hyperthyroidism.
- Use any of the following medications: hypolipidemic agent, oral contraceptive, antihypertensive agents, anabolic steroids, diuretics.

What will happen to me if I decide to take part in the study?

You will come to the Functional Food Centre at Oxford Brookes University for seven visits at least 1 week apart.

The first visit will be a screening visit to find out your eligibility to take part in the study by measuring your LDL-cholesterol level. If the result is between the levels of 2.58mmol/l and 4.11mmol/l then you will be included in the study. After that you will be assigned either to low or high dose of lecithin, which will be provided to you to be taken daily for four weeks (the direction of consuming will be explained to you later by the researcher). Blood and anthropometric measurement will be taken at baseline, after 2 weeks and at the end of the intervention.

After discontinuing the lecithin supplementation you will continue visiting FFC every week to have blood measurements to find the washout period of lecithin on the lipid profile on blood stream (when LDL levels return to pre-supplementation values).

During the study we will encourage you to stick to your usual diet and physical activity.

Visit 1: at the start of your first visit, you will be asked to sign a consent form and then the researcher will record some basic information and assess your eligibility to take part. After that we will need to take a few drops of blood using a finger prick method (one prick) to measure your lipid profile including LDL-cholesterol level. If the result of LDL-cholesterol was more than 2.58mmol/l (the results will be known immediately at the same visit) than you will be eligible to take part otherwise you will not be able to take part (if lower than 2.58mmol/l or higher than 4.11mmol/l).

If you are eligible and you would like to continue the study we will:

- 1- Take your anthropometric measurements including: weight, height, body fat percentage and body mass index.
- 2- Give you a 4 day diet record for you to complete so that we can estimate your daily intake of fat and cholesterol (you can complete it at home and bring it with you at the next visit).
- 3- Complete a physical activity chart to find your activity level (this will be done by the researcher)
- 4- And you will be randomly assigned to one of two groups: low dose or high dose lecithin supplementation and you will be provided with lecithin to start consuming every day for one month. Instruction when and how to use will be explained to you.

Visit 2: 2 weeks later we will take anthropometric measurements and finger prick blood sample (one prick) and provide you with a further 2 weeks supply of lecithin.

Visit 3: after a further 2 weeks we will retake anthropometric measurements and finger prick blood sample (one prick) and discontinue the lecithin supplementation.

Visit 4, 5, 6 and 7: each visit will be one week apart you will visit FFC for anthropometric and blood measurements. You will not need to take anything but you will be asked to try and maintain your normal diet and exercise habits. (Please see table 1 at end)

Are there any possible disadvantages or risk if I choose to take part?

There are limited risks in participating in this study. However we will need to take blood samples at each visit (seven visits) which may give some discomfort, but these will be taken by a trained researcher.

lecithin supplementations may cause in a rare cases some occasional diarrhea, abdominal cramps and bloating but we will inform you to contact us directly if you had any unusual symptoms and maybe you will be withdrawn immediately.

What are the possible benefits of taking part?

- You will be able to have your lipid profile measure.
- You might improve your LDL and cholesterol level as a result of the lecithin supplement intake.
- You will receive a report detailing your baseline anthropometric values and any changes occurring during the one month of lecithin supplementation.
- You will receive report of any lipid profile changes.
- At the end of the study you will be given a £50 amazon voucher as a thank you for your time.

Will the study results be kept confidential?

Your right to privacy will be respected. All information collected about you during this study will be kept strictly confidential, subject to legal limitation. The information about you will be stored on a secure password protected database, only accessible by the research team. You will

not be identified individually in any written or spoken presentation of the study. You will be allocated a study number which will be used as a code to identify you on the study database and kept securely for a period of 10 years after the study has finished, after then the information will be destroyed.

What will happen to the results of the research study?

The results will be presented as a part of a PhD thesis and in scientific poster or journals however, all data published will be anonymised.

Who is organising and funding the research?

The research is being conducted under the supervision of Dr Catherine Kerr and Dr Helen Lightowler at the Department of Sport and Health Sciences at Oxford Brookes University.

Who has reviewed the study?

This research study has been reviewed and approved by the University Research Ethics Committee (UREC). If you have any concerns about how this study has been conducted you can contact the University Research Ethics Chair on ethics@brookes.ac.uk

Contact for further information

You can contact the researchers at any time if you have any questions or concerns

Ms Majd Jan email: 14097361@brookes.ac.uk

Dr Catherine Kerr email: catherine.kerr@brookes.ac.uk

Dr Helen Lightowler email: hlightowler@brookes.ac.uk

Thank you for your time to read this information sheet.

Table 1:

(First visit) Explain the study and answer any question - then sign consent form □ Screening test Measure LDL-cholesterol by finger prick □ ≥ 2.58 mmol and ≤ 4.11 mmol included < 2.58 mmol and > 4.11 mmol excluded (Participant are free either to start the study or can discuss the result with their GP and then can come back to take part if they like)	
week 0	Blood measurements (baseline) Randomly blind assigned to one of two groups, either: □ □ Group 1 (low dose 600mg) Group 2 (high dose 2400mg) Collect lecithin supplements Start the intervention (Lecithin supplements)
□ □ 2 weeks	
week 2	(Second visit) Blood measurements Collect lecithin supplements
□ □ 2 weeks	
week 4	(Third visit) Blood measurements End of intervention
□ □ 1 week	
week 5	(Fourth visit) Blood measurements
□ □ 1 week	
Week 6	(Fifth visit) Blood measurements
□ □ 1 week	
week 7	(Sixth visit) Blood measurements
□ □ 1 week	
week 8	(Seventh visit) Blood measurements ~End~

Consent Form

Dietary phosphatidylcholine assessed by food-frequency questionnaire in relation to lipid biomarkers in healthy populations

Majd Jan, PhD researcher 14097361@brookes.ac.uk
Dr Helen Lightowler, Supervisor hlightowler@brookes.ac.uk
Dr Sangeetha Thondre, Supervisor pthondre@brookes.ac.uk
Dr Alaaddine El-Chab, Supervisor a.el-chab@brookes.ac.uk

Please initial box

I confirm that I have read and understand the information sheet for the above study and have had the opportunity to ask questions.

I understand that my participation is voluntary and that I am free to withdraw at any time, without giving reason.

I understand that my information and measurements given will be de-identified in any publication and reports.

I agree to take part in the above study and give blood samples.

Please tick box

I agree that the data may be stored after it has been de-identified and may be used in future research publications. Yes No

Name of Participant	Date	Signature
-----	-----	-----

Name of Researcher	Date	Signature
-----	-----	-----

Consent Form

The effects of dietary lecithin on cholesterol and lipid profile

Majd Jan, PhD researcher (Human Nutrition)

14097361@brookes.ac.uk

Dr. Helen Lightowler, Supervisor

hlightowler@brookes.ac.uk

Please initial box

I confirm that I have read and understand the information sheet for the above study and have had the opportunity to ask questions.

I understand that my participation is voluntary and that I am free to withdraw at any time, without giving reason.

I understand that my information that I give will only be used anonymously and I will not be identified when my view are presented to other participants or in any publication and reports.

I agree to take part in the above study.

Please tick box

I agree that the data may be stored after it has been anonymised and may be used in future research publications.

Yes No

Name of Participant

Date

Signature

Name of Researcher

Date

Signature

Appendix 4a

Basic Data Sheet

Dietary phosphatidylcholine intake assessed by food frequency questionnaire in relation to lipid biomarkers

Participant name:	Candidate code:	Age:
Gender: ☐ Male ☐ Female	Date of birth:	
Contact telephone:		
Email:		
Ethnicity: ☐ White ☐ Asian/Asian British ☐ Black/ African/Caribbean/Black British ☐ Mixed/Multiple ethnic groups ☐ Arab		
Smoking status: ☐ smoker – never ☐ Smoker- past ☐ smoking < 10 per day ☐ smoking 11 – 20 per day ☐ smoking >20 per day		
Do you have any medical condition? ☐ No ☐ Yes If yes, can you provide more details?		
Medications:		
Have you been taking any dietary supplements through the last month? ☐ No ☐ Yes If yes, can you provide more details?		

Basic Data Sheet

Participant name:	Candidate code:	Age:
Gender: ☐ Male ☐ Female	Nationality:	
Contact telephone:	Email:	
Ethnicity:		
☐ White ☐ Asian/Asian British ☐ Black/ African/Caribbean/Black British groups ☐ Mixed/Multiple ethnic ☐ Arab		
Physical Activity:		
☐ Sedentary ☐ Light active ☐ Moderate active ☐ Very active		
Smoking status:		
☐ Non-smoker ☐ Smoker if smoker how many per day -----		
Medical history:		
Are you under any of these medication:		
☐ Hypolipidemic agents ☐ Diuretics ☐ Oral contraceptive ☐ Antihypertensive agents ☐ Anabolic steroids ☐ None		
Are you taking any dietary supplements?		
☐ Yes ☐ No		

Appendix 5

Alcohol Consumption Assessment

1. In a typical month, how often do you have a drink containing alcohol? a) Never (0 points) b) Once a month or less (1 points) c) 2 to 4 times a month (2 points) d) 2 to 3 times a week (3 points) e) 4 or more times a week (4 points)	6. How often during the last year have you needed an alcoholic drink in the morning to get yourself going after a heavy drinking session? a) Never (0 points) b) Less than monthly (1 point) c) Monthly (2 points) d) Weekly (3 points) e) Daily or almost daily (4 points)
2. How many units of alcohol do you have on a typical day when you are drinking? a) 1-2 (0 points) b) 3-4 (1 point) c) 5-6 (2 points) d) 7-8 (3 points) e) 9+ (4 points)	7. How often during the last year have you had a feeling of guilt or remorse after drinking? a) Never (0 points) b) Less than monthly (1 points) c) Monthly (2 points) d) Weekly (3 points) e) Daily or almost daily (4 points)
3. How often do you have six or more units on one occasion? a) Never (0 points) b) Less than monthly (1 points) c) Monthly (2 points) d) Weekly (3 points) e) Daily or almost daily (4 points)	8. How often during the last year have you been unable to remember what happened the night before because you had been drinking? a) Never (0 points) b) Less than monthly (1 point) c) Monthly (2 points) d) Weekly (3 points) e) Daily (4 points)
4. How often during the last year have you failed to do what was normally expected from you because of your drinking? a) Never (0 points) b) Less than monthly (1 points) c) Monthly (2 points) d) Weekly (3 points) e) Daily or almost daily (4 points)	9. Have you or somebody else been injured as a result of your drinking? a) No, this has never happened (0 points) b) Yes, but not in the past year (2 points) c) Yes, during the past year (4 points)
5. How often during the last year have you found that you were not able to stop drinking once you had started? a) Never (0 points) b) Less than monthly (1 points) c) Monthly (2 points) d) Weekly (3 points) e) Daily or almost daily (4 points)	10. Has a relative, friend, doctor or health worker been concerned about your drinking or suggested you cut down? a) No, never (0 points) b) Yes, but not in the past year (2 points) c) Yes, during the past year (4 points)

This is one unit of alcohol...



Half pint of
regular beer,
lager or cider



1 small glass
of wine



1 single
measure
of spirits



1 small
glass of
sherry



1 single
measure
of aperitifs

...and each of these is more than one unit



Pint of Regular
Beer/Lager/Cider



Pint of Premium
Beer/Lager/Cider



Alcopop or
can/bottle of
Regular Lager



Can of Premium
Lager
or Strong Beer



Can of Super
Strength
Lager



Glass of Wine
(175ml)

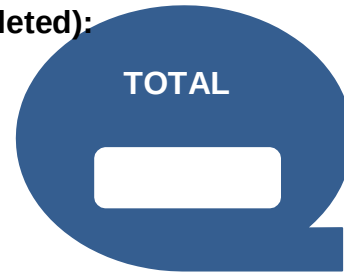


Bottle of
Wine

STANDARD DRINK EQUIVALENTS	APPROXIMATE NUMBER OF STANDARD DRINKS IN:
BEER or COOLER	
12 oz.  ~5% alcohol	12 oz. = 1 16 oz. = 1.3 22 oz. = 2 40 oz. = 3.3
MALT LIQUOR	
8-9 oz.  ~7% alcohol	12 oz. = 1.5 16 oz. = 2 22 oz. = 2.5 40 oz. = 4.5
TABLE WINE	
5 oz.  ~12% alcohol	a 750 mL (25 oz.) bottle = 5
80-proof SPIRITS (hard liquor)	
1.5 oz.  ~40% alcohol	a mixed drink = 1 or more* a pint (16 oz.) = 11 a fifth (25 oz.) = 17 1.75 L (59 oz.) = 39 *Note: Depending on factors such as the type of spirits and the recipe, one mixed drink can contain from one to three or more standard drinks.

TOTAL AUDIT Score (all 10 questions completed):

0 – 7 Lower risk,
8 – 15 Increasing risk,
16 – 19 Higher risk,
20+ Possible dependence



RESULTS:

0-7 points

Based on your answers today you're drinking in a way that is sociable and is unlikely to harm your health.

As long as your drinking does remain within recommended levels, you are considered a 'lower-risk' drinker as there is only a low risk that the way you drink will contribute to future health problems.

The NHS recommends that women should not regularly drink more than 2-3 units a day and men should not regularly drink more than 3-4 units a day.

Drinking consistently within these limits is called 'lower-risk' rather than 'safe' because drinking alcohol is never completely safe.

Remember, there can be risks from one-off episodes of heavy drinking too.

8-19 points

Based on your answers today you are drinking at harmful levels.

The NHS recommends that women should not regularly drink more than 2-3 units a day and men should not regularly drink more than 3-4. If you drink every day, it's recommended that you have at least two alcohol-free days a week.

Alcohol affects all parts and systems of the body and if you are regularly drinking above the guidelines, your risk of developing a serious illness is higher than non-drinkers. This includes cancer of the mouth, neck and throat, breast cancer in women, liver cirrhosis and high blood pressure.

At these levels of drinking, you may already have alcohol-related problems, such as fatigue or depression, weight gain, poor sleep and sexual problems. You're probably in worse physical shape than you would be otherwise. Also, you could easily have higher blood pressure due to your drinking.

Some people argue a lot when they drink, which can negatively affect their relationships with family and friends.

20-40 points

Based on your answers today you are drinking at harmful levels and your drinking is already causing you problems.

The NHS recommends that women should not regularly drink more than 2-3 units a day and men should not regularly drink more than 3-4 units a day. If you drink every day, it's recommended that you have at least two alcohol-free days a week.

You have a much higher risk of developing alcohol-related health problems. Your body has probably suffered some damage already, even if you're not yet aware of it.

INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE (October 2002)

LONG LAST 7 DAYS SELF-ADMINISTERED FORMAT FOR USE WITH YOUNG AND MIDDLE-AGED ADULTS (15-69 years)

The International Physical Activity Questionnaires (IPAQ) comprises a set of 4 questionnaires. Long (5 activity domains asked independently) and short (4 generic items) versions for use by either telephone or self-administered methods are available. The purpose of the questionnaires is to provide common instruments that can be used to obtain internationally comparable data on health-related physical activity.

Background on IPAQ

The development of an international measure for physical activity commenced in Geneva in 1998 and was followed by extensive reliability and validity testing undertaken across 12 countries (14 sites) during 2000. The final results suggest that these measures have acceptable measurement properties for use in many settings and in different languages, and are suitable for national population-based prevalence studies of participation in physical activity.

Using IPAQ

Use of the IPAQ instruments for monitoring and research purposes is encouraged. It is recommended that no changes be made to the order or wording of the questions as this will affect the psychometric properties of the instruments.

Translation from English and Cultural Adaptation

Translation from English is encouraged to facilitate worldwide use of IPAQ. Information on the availability of IPAQ in different languages can be obtained at www.ipaq.ki.se. If a new translation is undertaken we highly recommend using the prescribed back translation methods available on the IPAQ website. If possible please consider making your translated version of IPAQ available to others by contributing it to the IPAQ website. Further details on translation and cultural adaptation can be downloaded from the website.

Further Developments of IPAQ

International collaboration on IPAQ is on-going and an ***International Physical Activity Prevalence Study*** is in progress. For further information see the IPAQ website.

More Information

More detailed information on the IPAQ process and the research methods used in the development of IPAQ instruments is available at www.ipaq.ki.se and Booth, M.L. (2000). *Assessment of Physical Activity: An International Perspective*. Research Quarterly for Exercise and Sport, 71 (2): s114-20. Other scientific publications and presentations on the use of IPAQ are summarized on the website.

INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE

We are interested in finding out about the kinds of physical activities that people do as part of their everyday lives. The questions will ask you about the time you spent being physically active in the **last 7 days**. Please answer each question even if you do not consider yourself to be an active person. Please think about the activities you do at work, as part of your house and yard work, to get from place to place, and in your spare time for recreation, exercise or sport.

Think about all the **vigorous** and **moderate** activities that you did in the **last 7 days**.

Vigorous physical activities refer to activities that take hard physical effort and make you breathe much harder than normal. **Moderate** activities refer to activities that take moderate physical effort and make you breathe somewhat harder than normal.

PART 1: JOB-RELATED PHYSICAL ACTIVITY

The first section is about your work. This includes paid jobs, farming, volunteer work, course work, and any other unpaid work that you did outside your home. Do not include unpaid work you might do around your home, like housework, yard work, general maintenance, and caring for your family. These are asked in Part 3.

1. Do you currently have a job or do any unpaid work outside your home?

☐

Yes

☐

No



Skip to PART 2: TRANSPORTATION

The next questions are about all the physical activity you did in the **last 7 days** as part of your paid or unpaid work. This does not include traveling to and from work.

2. During the **last 7 days**, on how many days did you do **vigorous** physical activities like heavy lifting, digging, heavy construction, or climbing up stairs **as part of your work**? Think about only those physical activities that you did for at least 10 minutes at a time.

_____ **days per week**

☐

No vigorous job-related physical activity



Skip to question 4

3. How much time did you usually spend on one of those days doing **vigorous** physical activities as part of your work?

_____ **hours per day**

_____ **minutes per day**

4. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **moderate** physical activities like carrying light loads **as part of your work**? Please do not include walking.

_____ **days per week**

☐

No moderate job-related physical activity



Skip to question 6

5. How much time did you usually spend on one of those days doing **moderate** physical activities as part of your work?

_____ **hours per day**
_____ **minutes per day**

6. During the **last 7 days**, on how many days did you **walk** for at least 10 minutes at a time **as part of your work**? Please do not count any walking you did to travel to or from work.

_____ **days per week**

☐

No job-related walking



Skip to PART 2: TRANSPORTATION

7. How much time did you usually spend on one of those days **walking** as part of your work?

_____ **hours per day**
_____ **minutes per day**

PART 2: TRANSPORTATION PHYSICAL ACTIVITY

These questions are about how you traveled from place to place, including to places like work, stores, movies, and so on.

8. During the **last 7 days**, on how many days did you **travel in a motor vehicle** like a train, bus, car, or tram?

_____ **days per week**

☐

No traveling in a motor vehicle



Skip to question 10

9. How much time did you usually spend on one of those days **traveling** in a train, bus, car, tram, or other kind of motor vehicle?

_____ **hours per day**
_____ **minutes per day**

Now think only about the **bicycling** and **walking** you might have done to travel to and from work, to do errands, or to go from place to place.

10. During the **last 7 days**, on how many days did you **bicycle** for at least 10 minutes at a time to go **from place to place**?

_____ **days per week**

☐

No bicycling from place to place



Skip to question 12

11. How much time did you usually spend on one of those days to **bicycle** from place to place?

_____ **hours per day**
_____ **minutes per day**

12. During the **last 7 days**, on how many days did you **walk** for at least 10 minutes at a time to go **from place to place**?

_____ **days per week**

☐

No walking from place to place



***Skip to PART 3: HOUSEWORK,
HOUSE MAINTENANCE, AND
CARING FOR FAMILY***

13. How much time did you usually spend on one of those days **walking** from place to place?

_____ **hours per day**
_____ **minutes per day**

PART 3: HOUSEWORK, HOUSE MAINTENANCE, AND CARING FOR FAMILY

This section is about some of the physical activities you might have done in the **last 7 days** in and around your home, like housework, gardening, yard work, general maintenance work, and caring for your family.

14. Think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **vigorous** physical activities like heavy lifting, chopping wood, shoveling snow, or digging **in the garden or yard**?

_____ **days per week**

☐

No vigorous activity in garden or yard



Skip to question 16

15. How much time did you usually spend on one of those days doing **vigorous** physical activities in the garden or yard?

_____ **hours per day**
_____ **minutes per day**

16. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **moderate** activities like carrying light loads, sweeping, washing windows, and raking **in the garden or yard**?

_____ **days per week**

☐

No moderate activity in garden or yard



Skip to question 18

LONG LAST 7 DAYS SELF-ADMINISTERED version of the IPAQ. Revised October 2002.

17. How much time did you usually spend on one of those days doing **moderate** physical activities in the garden or yard?

_____ hours per day
_____ minutes per day

18. Once again, think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **moderate** activities like carrying light loads, washing windows, scrubbing floors and sweeping **inside your home**?

_____ days per week

☐

No moderate activity inside home



***Skip to PART 4: RECREATION,
SPORT AND LEISURE-TIME
PHYSICAL ACTIVITY***

19. How much time did you usually spend on one of those days doing **moderate** physical activities inside your home?

_____ hours per day
_____ minutes per day

PART 4: RECREATION, SPORT, AND LEISURE-TIME PHYSICAL ACTIVITY

This section is about all the physical activities that you did in the **last 7 days** solely for recreation, sport, exercise or leisure. Please do not include any activities you have already mentioned.

20. Not counting any walking you have already mentioned, during the **last 7 days**, on how many days did you **walk** for at least 10 minutes at a time **in your leisure time**?

_____ days per week

☐

No walking in leisure time



Skip to question 22

21. How much time did you usually spend on one of those days **walking** in your leisure time?

_____ hours per day
_____ minutes per day

22. Think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **vigorous** physical activities like aerobics, running, fast bicycling, or fast swimming **in your leisure time**?

_____ days per week

☐

No vigorous activity in leisure time



Skip to question 24

LONG LAST 7 DAYS SELF-ADMINISTERED version of the IPAQ. Revised October 2002.

23. How much time did you usually spend on one of those days doing **vigorous** physical activities in your leisure time?

_____ **hours per day**
_____ **minutes per day**

24. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **moderate** physical activities like bicycling at a regular pace, swimming at a regular pace, and doubles tennis **in your leisure time**?

_____ **days per week**

☐

No moderate activity in leisure time



Skip to PART 5: TIME SPENT SITTING

25. How much time did you usually spend on one of those days doing **moderate** physical activities in your leisure time?

_____ **hours per day**
_____ **minutes per day**

PART 5: TIME SPENT SITTING

The last questions are about the time you spend sitting while at work, at home, while doing course work and during leisure time. This may include time spent sitting at a desk, visiting friends, reading or sitting or lying down to watch television. Do not include any time spent sitting in a motor vehicle that you have already told me about.

26. During the **last 7 days**, how much time did you usually spend **sitting** on a **weekday**?

_____ **hours per day**
_____ **minutes per day**

27. During the **last 7 days**, how much time did you usually spend **sitting** on a **weekend day**?

_____ **hours per day**
_____ **minutes per day**

This is the end of the questionnaire, thank you for participating.

Appendix 6b

INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE (August 2002)

SHORT LAST 7 DAYS SELF-ADMINISTERED FORMAT

FOR USE WITH YOUNG AND MIDDLE-AGED ADULTS (15-69 years)

The International Physical Activity Questionnaires (IPAQ) comprises a set of 4 questionnaires. Long (5 activity domains asked independently) and short (4 generic items) versions for use by either telephone or self-administered methods are available. The purpose of the questionnaires is to provide common instruments that can be used to obtain internationally comparable data on health-related physical activity.

Background on IPAQ

The development of an international measure for physical activity commenced in Geneva in 1998 and was followed by extensive reliability and validity testing undertaken across 12 countries (14 sites) during 2000. The final results suggest that these measures have acceptable measurement properties for use in many settings and in different languages, and are suitable for national population-based prevalence studies of participation in physical activity.

Using IPAQ

Use of the IPAQ instruments for monitoring and research purposes is encouraged. It is recommended that no changes be made to the order or wording of the questions as this will affect the psychometric properties of the instruments.

Translation from English and Cultural Adaptation

Translation from English is supported to facilitate worldwide use of IPAQ. Information on the availability of IPAQ in different languages can be obtained at www.ipaq.ki.se. If a new translation is undertaken we highly recommend using the prescribed back translation methods available on the IPAQ website. If possible please consider making your translated version of IPAQ available to others by contributing it to the IPAQ website. Further details on translation and cultural adaptation can be downloaded from the website.

Further Developments of IPAQ

International collaboration on IPAQ is on-going and an *International Physical Activity Prevalence Study* is in progress. For further information see the IPAQ website.

More Information

More detailed information on the IPAQ process and the research methods used in the development of IPAQ instruments is available at www.ipaq.ki.se and Booth, M.L. (2000). *Assessment of Physical Activity: An International Perspective*. Research Quarterly for Exercise and Sport, 71 (2): s114-20. Other scientific publications and presentations on the use of IPAQ are summarized on the website.

INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE

We are interested in finding out about the kinds of physical activities that people do as part of their everyday lives. The questions will ask you about the time you spent being physically active in the **last 7 days**. Please answer each question even if you do not consider yourself to be an active person. Please think about the activities you do at work, as part of your house and yard work, to get from place to place, and in your spare time for recreation, exercise or sport.

Think about all the **vigorous** activities that you did in the **last 7 days**. **Vigorous** physical activities refer to activities that take hard physical effort and make you breathe much harder than normal. Think *only* about those physical activities that you did for at least 10 minutes at a time.

1. During the **last 7 days**, on how many days did you do **vigorous** physical activities like heavy lifting, digging, aerobics, or fast bicycling?

_____ **days per week**

☐ No vigorous physical activities → **Skip to question 3**

2. How much time did you usually spend doing **vigorous** physical activities on one of those days?

_____ **hours per day**

_____ **minutes per day**

☐ Don't know/Not sure

Think about all the **moderate** activities that you did in the **last 7 days**. **Moderate** activities refer to activities that take moderate physical effort and make you breathe somewhat harder than normal. Think *only* about those physical activities that you did for at least 10 minutes at a time.

3. During the **last 7 days**, on how many days did you do **moderate** physical activities like carrying light loads, bicycling at a regular pace, or doubles tennis? Do not include walking.

_____ **days per week**

☐ No moderate physical activities → **Skip to question 5**

4. How much time did you usually spend doing **moderate** physical activities on one of those days?

_____ **hours per day**

_____ **minutes per day**

☐ Don't know/Not sure

Think about the time you spent **walking** in the **last 7 days**. This includes at work and at home, walking to travel from place to place, and any other walking that you have done solely for recreation, sport, exercise, or leisure.

5. During the **last 7 days**, on how many days did you **walk** for at least 10 minutes at a time?

_____ **days per week**

☐ No walking → ***Skip to question 7***

6. How much time did you usually spend **walking** on one of those days?

_____ **hours per day**

_____ **minutes per day**

☐ Don't know/Not sure

The last question is about the time you spent **sitting** on weekdays during the **last 7 days**. Include time spent at work, at home, while doing course work and during leisure time. This may include time spent sitting at a desk, visiting friends, reading, or sitting or lying down to watch television.

7. During the **last 7 days**, how much time did you spend **sitting** on a **week day**?

_____ **hours per day**

_____ **minutes per day**

☐ Don't know/Not sure

This is the end of the questionnaire, thank you for participating.

Appendix 7

Weighed Food Diary record

Thank you for participating in this study. This sheet is a way of recording your food intake. It is important that you record every single item that you have consumed otherwise the record and the results will not be reliable.

Principles and useful tips for making a perfect Weighed Food Diary record

- 1 Record **EVERYTHING** you have eaten, might it be those two-three jelly beans you have been offered by your child or food supplements such as vitamins, minerals, cod liver oil, etc.
 - 2 Record the food items and their amounts **in a real time**, do not wait until evening, otherwise you will forget some items and the record and the analysis will be unreliable.
 - 3 Record left-overs when you are able to record them, even at the end of the day or the next day, if they did not dry out (affecting the reading of weight).
 - 4 Note down the **brand** and **name** of the product if not made by yourself. Some **photos** of the wrap or package and information about the product on it may become handy if it was a less common item.
 - 5 Make sure you have noted down the **correct portion size**. For example, the crisps packs exist in at least three sizes. The volume of fluids is also written on their bottle or carton. Bread slices can be of different thickness and sizes, too. If not possible to weigh them, then specify the product as much as you can so that I can find it in the database or elsewhere.
 - 6 The nature of the preparation/cooking method of food or snack is also important: fried or baked crisps? Deep fried Margherita pizza or a stone baked style? Do not forget to specify the size of pizza, even if only approximately.
 - 7 You can send me the photos of the product package or the whole meal you have had on your plate. Some details such as the ingredient or the Nutrition Information may also become handy for some less known items.
- **Important:** do not try to alter your dietary habits and the food choices just because you are recording, to make it easier for you. It will not represent your usual diet.

Having said that, you should avoid recording during feasts such as Birthdays or Christmas day, for exactly the same reason.

That is all. The main rule is to record to as much detail as possible and to record everything you consumed in as precise amounts as possible. It is better to provide rather more details than too little.

On the next page is an example of one of my Food diary records.

Happy recording and if you have any questions, please send me an e-mail to 14097361@brookes.ac.uk

Example:

time	Place	Description of food	brand	amount	Leftover
6.30 am	Kitchen Alone	Filter coffee decaffeinated milk (fresh, semi-skimmed) Sugar white	Douwe Egberts Silverspoon	400ml 5ml 7gm	/
7.30 am	Kitchen Partner TV on At table	Cornflakes Milk (fresh, semi-skimmed) Toast, granary medium sliced Light spread Marmalade	Tesco's own Hovis Flora Hartleys	45gm 20ml 30gm 30gm 20gm	/
10.15 am	Office desk Alone	Instant coffee, not decaffeinated Milk (fresh, whole) Sugar brown	Kenco	400ml 5ml 7gm	/
11 am	Office desk Alone	Digestive biscuit – chocolate coated on one side	McVities	60gm	/
12.30 pm	12.30 pm Work tea room With colleagues	Ham Bread, wholemeal, thick sliced Light spread Low fat Mayonnaise Smoked ham thinly sliced Lettuce, iceberg Cucumber with skin Unsweetened orange juice from canteen	Tesco's own Flora Hellmans Tesco's own Tropicana	2 slices thin (40gm) 60gm Spread (10gm) 20gm 2 slices 40gm 6gm 4 thin slices 10gm 250ml carton	/

3 pm	Meeting room With supervisor variety	Tea, decaffeinated Milk (fresh, whole) Jaffa cake – mini	Twinnings Tesco's own McVities	300ml 10ml 6 (50gm)	/
6.30 pm	Pub, partner	Gin Lager Salted peanuts	Gordon's Draught KP	Single measure 1 pint 1 handful	/
8 pm	Dining room Family TV off At table	Spaghetti, wholemeal, cooked Bolognese sauce (see recipe) Courgettes (fried in butter) Tinned peaches in juice (juice drained) Single cream UHT Orange squash No Added Sugar	Tesco's own Prince's Sainsbury's own	100gm 6 tablespoons (45gm) 4 tablespoons (40gm) 3 halves 15gm 1 tablespoon (7gm) 200ml glass, 1 part squash, 3 parts tap water	/ 30ml
9 pm	Sitting room Alone Not at table	Grapes, green, seedless Chocolates, chocolate creams Potato crisps, Prawn Cocktail	Bendicks Walkers	15 (60gm) 29gm 25g bag from multipack	5gm
10.30 pm	Bed room Partner No TV	Camomile tea (no milk or sugar)	Twinnings	300ml	/

Day 1:

time	Place	Description of food	brand	amount	Leftover

Day 2:

time	Place	Description of food	brand	amount	Leftover

Day 3:

time	Place	Description of food	brand	amount	Leftover

Day 4:

time	Place	Description of food	brand	amount	Leftover

Day 5:

time	Place	Description of food	brand	amount	Leftover

Appendix 8

Food Frequency Questionnaire of Phosphatidylcholine and Saturated fatty acids

Participant code:

- For each food listed, check the box indicating how often, on average, you have used the amount specified during the last year.

Foods item		Servin g size	6+ per day	4-5 per day	2-3 per day	1 per day	5-6 per week	2-4 per week	1 per week	1-3 per month	Almost never
Dairy and egg											
1.	Milk, whole	1 cup									
2.	Milk, 2% fat	1 cup									
3.	Milk, skim	1 cup									
4.	Coconut milk	1 cup									
5.	Soy milk	1 cup									
6.	Yogurt, plain	1 cup									
7.	Yogurt, fruit	1 cup									
8.	Yogurt, fruit, non-fat	1 cup									
9.	Cottage cheese	1oz.									
10.	Cheddar cheese	1oz.									

Foods item		Servin g size	6+ per day	4-5 per day	2-3 per day	1 per day	5-6 per week	2-4 per week	1 per week	1-3 per month	Almost never
11.	Cream cheese	1 Tbsp.									
12.	Sour cream	1 Tbsp.									
13.	Butter with salt	1 Tbsp.									
14.	Eggs, whole	1 med									
15.	Egg, yolk	1 yolk									
Breakfast cereals and grains											
16.	Amaranth	1 cup									
17.	Barley malt flour	1cup									
18.	Brown rice, cooked	1cup									
19.	Egg noodles	1cup									
20.	Oat bran raw	1cup									

Foods item		Servin g size	6+ per day	4-5 per day	2-3 per day	1 per day	5-6 per week	2-4 per week	1 per week	1-3 per month	Almost never
21.	Plain oats	½ cup									
22.	Quinoa cooked	1 cup									
23.	Wheat germ	1 cup									
24.	White Rice, cooked	1 cup									
Fruits											
25.	Apples	1 med									
26.	Apricot, dried	1 med									
27.	Avocados	1 med									
28.	Bananas	1 med									
29.	Blueberries	1 cup									
30.	Cantaloupe	1 med slice									
31.	Grapefruit	1 med									
32.	Kiwi	1 med									
33.	Oranges	1 med									

Foods item		Serving size	6+ per day	4-5 per day	2-3 per day	1 per day	5-6 per week	2-4 per week	1 per week	1-3 per month	Almost never
34.	Peaches	1 med									
35.	Pears	1 med									
36.	Prunes, dried	1 med									
37.	Raspberries	1 cup									
38.	Strawberries	1 cup									
39.	Mandarins	1 med									
40.	Orange juice	1cup									
41.	Apple juice	1cup									
Vegetables											
42.	Edamame	1 cup									
43.	Artichokes	1 med									
44.	Asparagus	4 spear									
45.	Beets, canned	1 med									

Foods item		Serving size	6+ per day	4-5 per day	2-3 per day	1 per day	5-6 per week	2-4 per week	1 per week	1-3 per month	Almost never
46.	Broccoli	1 cup									
47.	Brussels sprouts	1 sprout									
48.	Cabbage	1 cup									
49.	Carrots, raw	1 med									
50.	Cauliflower	1 cup									
51.	Baked potatoes	1 med									
52.	Mushrooms	1 med									
53.	Onions, sautéed	1 cup									
54.	Peas, frozen	1 cup									
55.	Green beans	1 cup									
56.	Spinach	1 cup									
57.	Sweet pepper	1 med									
58.	Sweet potatoes	1 med									
59.	Yellow corn	1 cup									
60.	Zucchini	1 med									

Foods item		Serving size	6+ per day	4-5 per day	2-3 per day	1 per day	5-6 per week	2-4 per week	1 per week	1-3 per month	Almost never
Legumes and legume products											
61.	Beans, kidney	1 cup									
62.	Beans, navy	1cup									
63.	Soybean, raw	1cup									
64.	tofu, soft	1 cube									
Chickens											
65.	Chicken hot dogs	3 oz.									
66.	Chicken liver	3 oz.									
67.	Chicken roasted (no skin)	3 oz.									
68.	Chicken roasted (w/skin)	3 oz.									

Foods item		Servin g size	6+ per day	4-5 per day	2-3 per day	1 per day	5-6 per week	2-4 per week	1 per week	1-3 per month	Almost never
Beef products											
69.	Liver, pan fried	3 oz.									
70.	Steak	3 oz.									
71.	Minced beef	3 oz.									
Pork products											
72.	Pork loin cooked	3 oz.									
73.	Pork sausage	3 oz.									
74.	Bacon	3 oz.									
Finfish and shellfish											
75.	Shrimp	3 oz.									
76.	Fish- atlantic cod	3 oz.									
77.	Salmon	3 oz.									
78.	Fish stick	3 oz.									
79.	Tuna	3 oz.									

Foods item		Servin g size	6+ per day	4-5 per day	1 per day	5-6 per week	2-4 per week	1-3 per month	Almost never
Soups									
80.	Chicken noodle soup	1 cup							
81.	Hot and sour soup	1 cup							
82.	New England clam chowder soup	1 cup							
Sauce and fat									
83.	Ketchup	1 Tbsp.							
84.	Tomato sauce	1 Tbsp.							
85.	Tomato paste	1 Tbsp.							
86.	Mayonnaise, low fat	1 Tbsp.							
87.	Mayonnaise, regular	1 Tbsp.							
88.	Peanut butter	1 Tbsp.							
89.	Soy oil	1 Tbsp.							

Foods item		Servin g size	6+ per day	4-5 per day	2-3 per day	1 per day	5-6 per week	2-4 per week	1 per week	1-3 per month	Almost never
Spices and herbs											
90.	Chilli powder	1 tsp									
91.	Curry powder	1 tsp									
92.	Mustard seeds	1 tsp									
93.	Parsley dried	1 tsp									
94.	Turmeric	1 tsp									
Nuts and seed											
95.	Almond	2 oz.									
96.	Cashew	2 oz.									
97.	Flax seed	2 oz.									
98.	Peanuts	2 oz.									
99.	Pecans	2 oz.									
100.	Pine nut	2 oz.									
101.	Pistachio	2 oz.									
102.	Walnut	2 oz.									

Foods item		Serving size	6+ per day	4-5 per day	2-3 per day	1 per day	5-6 per week	2-4 per week	1 per week	1-3 per month	Almost never
Baked products											
103.	Wheat bread	1slice									
104.	White bread	1 slice									
105.	Chocolate chip cookie	1 cookie									
106.	Tortillas	1 tortilla									
107.	English muffins	1 muffin									
108.	Wheat cracker	1 cracker									
109.	Plain cracker	1 cracker									
110.	Danish pastry	1 pastry									
111.	Apple pie	1 piece									
112.	Chocolate cake, prepared	2 oz.									
113.	Pancakes plain	1 pancake									

Foods item		Serving size	6+ per day	4-5 per day	2-3 per day	1 per day	5-6 per week	2-4 per week	1 per week	1-3 per month	Almost never
114.	Frosted cake	67gm									
115.	Plain muffins	1muffin									
116.	Blueberry muffins	1 muffin									
Sugar and sweets and snacks											
117.	Ice cream	½ cup									
118.	Vanilla shake	16 fl oz.									
119.	Frozen yogurt	½ cup									
120.	Milk chocolate	1 bar									
121.	Snickers candy bar	1bar									
122.	Corn chips	1oz									
123.	Crisps	1oz									
124.	Popcorn	1cup									
125.	Pretzel, plain, salted	1oz									
126.	Granola Bar	1 bar									

Foods item		Serving size	6+ per day	4-5 per day	2-3 per day	1 per day	5-6 per week	2-4 per week	1 per week	1-3 per month	Almost never
Fast food											
127.	Cheese pizza	1slice									
128.	Chicken nuggets	4 pieces									
129.	Regular hamburger	1sandwic h									
130.	Tacos	1 taco									
131.	Lasagne with meat	15 oz.									
132.	Hotdog and bun	1 roll									
133.	Nachos with cheese	4 oz.									
134.	Potato chips	10 strips									
135.	Mashed potato	1 cup									
136.	Hash browns	1 piece									

This is end of the questionnaire.

Thank you!

Appendix 9

Daily Diet Record

Participant code: _____

Please record all food and beverages you consume in details for 4 days including portion size, food brand, preparation methods and if there is any leftover) the first page give as a an example:

Day: Wednesday		Date: 1 November 2015	
Food / beverage type	Cooking and preparation	Serving size	Leftover
Breakfast time: 7:00 am			
Toast	Toasted	2 slices	No
Sliced cheese (cheddar)	-	1 slice	No
Orange juice	Fresh	1 cup	¼ of the cup
Water	Tap water	1 cup	No
Snack time: 9:00am			
Crisp	-	Small pack	No
Tea	-	25gm	No
		1 cup	
Lunch time: 12:00pm			
White long grain rice	Boiled	10 table spoon	No
Chicken breast	Grilled, no skin	4 finger piece	No
Green salad (lettuce, cucumber)	Raw	size	No
Apple juice	Fresh juice	1 cup mix	No
		1 cup	
Snack time: 3:00pm			
Strawberries	Raw, fresh	4	No
Dinner time: 7:00pm			
Potato with cheddar cheese and corn	Baked	1 big potato	No
		1 table spoon cheese	
		2 table spoon corn	
water	Tap water	1 cup	No
Bed time snack time: 10pm			
Yoghurt	Low fat plain	1 cup	No
Water	Tap water	1 cup	No

Day one:

Day:		Date:	
Food / beverage type	Cooking and preparation	Serving size	Leftover
Breakfast		time:	
Snack		time:	
Lunch		time:	
Snack		time:	
Dinner		time:	
Bed time snack		time:	

Day two:

Day:		Date:	
Food / beverage type	Cooking and preparation	Serving size	Leftover
Breakfast		time:	
Snack		time:	
Lunch		time:	
Snack		time:	
Dinner		time:	
Bed time snack		time:	

Day three:

Day:		Date:	
Food / beverage type	Cooking and preparation	Serving size	Leftover
Breakfast		time:	
Snack		time:	
Lunch		time:	
Snack		time:	
Dinner		time:	
Bed time snack		time:	

Day four:

Day:		Date:	
Food / beverage type	Cooking and preparation	Serving size	Leftover
Breakfast		time:	
Snack		time:	
Lunch		time:	
Snack		time:	
Dinner		time:	
Bed time snack		time:	