The Fatty Acids-Inflammation Relationship Across The Lifecycle

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DEDICATION

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ABSTRACT

Dietary fatty acid intake, reflected by the endogenous fatty acid profile, has been associated with the pathogenesis and progression of cardiovascular diseases (CVD). Additional evidence is needed about the specific roles of individual fatty acids in the pathogenesis of inflammation, which is closely linked to CVD risk factors and interwined with oxidative stress and hemostatic dysfunction. It is also important to explore such relationships across the lifecycle. This dissertation, which includes four manuscripts, investigates the relations between fatty acids, biomarkers of inflammation (and oxidative stress and hemostasis), and cardiovascular health among different age groups. Specifically, the influences of adiposity and a genetic variant on the fatty acid/inflammation relations were also explored.

The first manuscript used data from a study of obesity, insulin resistance and CVD risk factors in adolescents. A cross-sectional analysis was conducted to examine whether overweight status modified the relations between serum phospholipid fatty acids from dairy fats (i.e. 15:0 and 17:0 fatty acids) and inflammation/oxidative stress among adolescents with a mean age of 15 years. Inverse associations were found between dairy fatty acids and three biomarkers of inflammation and oxidative stress among overweight adolescents, but not their normal weight counterparts. In additional analyses, we further examined the same study question on other fatty acids and observed similar effect modification of adiposity. Only in overweight adolescents, but not in normal adolescents, 18:0 and 20:3ω6 fatty acids were positively, while 20:4ω6 and 22:6ω3 fatty acids were inversely, related to inflammation/oxidative stress.

The second manuscript examined whether the cross-sectional relations between dietary intakes of polyunsaturated fatty acids (PUFA) and inflammation differed by genetic variant, peroxisome proliferator-activated receptor gamma (PPARγ) Pro12Ala polymorphism. A biracial cohort of middle-aged adults enrolled in the year-20 exam of the Coronary Artery Risk Development in Young Adults (CARDIA) study was studied. In women, higher dietary intakes of 20:4ω6, 20:5ω3 and 22:6ω3 fatty acids were related to lower levels of IL-6 (an inflammatory biomarker) among Ala allele carriers. In contrast, these PUFA/IL-6 relations were positive among male Ala carriers, and absent among female Pro homozygotes. Male Pro homozygotes who consumed more 20:5ω3 and 22:6ω3 fatty acids tended to have a lower IL-6 level.

The last two manuscripts were both prospective studies using data from the Atherosclerosis Risk in Communities (ARIC) study, which enrolled middle-aged adults. This cohort has been followed since year 1987-89.

Manuscript 3 examined the interactions between dietary fatty acid intake and inflammatory/hemostatic factors in relation to incident coronary heart disease (CHD) and ischemic stroke (IS). Dietary intakes of 18:2ω6 and 20:4ω6 fatty acids were found to modify the associations between serum albumin and incident CHD/IS. The prediction of low serum albumin level, a potential inflammatory biomarker, on incident CHD/IS was attenuated with increasing intake of 18:2ω6 fatty acid or decreasing intake of 20:4ω6 fatty acid.

Manuscript 4 included 3,715 ARIC participants enrolled at the Minnesota field center who had plasma phospholipid fatty acid measurements. In manuscript 4, the focuse was

to determine whether inflammation/hemostasis mediated the relation of phospholipid fatty acids with incident CHD and IS. Inflammation and hemostasis, represented by levels of factor VIIIc (VIIIc), white blood cell count (WBC) and fibrinogen, mediated the positive associations of 18:0 and 20:3ω6 fatty acids with incident CHD. A similar but less significant pattern was found for 16:1ω7 in relation to incident IS. Lower WBC, but not VIIIc or fibrinogen, partially explained the inverse relations of 17:0 and 20:4ω6 fatty acids with incident CHD.

In conclusion, this dissertation documents the associations between diverse fatty acids, inflammation and the development of CVD among different age-groups. The findings have enhanced the understanding of the health effects of individual fatty acids and the underlying mechanisms of fatty acid-inflammation-CVD relations, which are useful for advising food manufacturers and guiding CVD prevention.

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LIST OF ABBREVIATIONS

15-keto	15-keto-dihydro-PGF2α
AA	Arachidonic acid
AHA	American Heart Association
ALA	α-linolenic acid
Ala	Alanine
AMPK	AMP-activated protein kinase
ARIC	Atherosclerosis Risk in Communities study
BMI	Body mass index
CARDIA	Coronary Artery Risk Development in Young Adults study
CE	Cholesterol ester
CHD	Coronary heart disease
ChREBP	Carbohydrate responsive element binding protein
CI	Confidence interval
CLA	Conjugated linoleic acid
CRP	C-reactive protein
CT	Computed tomography
CVD	Cardiovascular disease
DGLA	Dihomo-γ-linolenic acid
DHA	Docosahexaenoic acid
ECG	Electrocardiogram
EDTA	Ethylenediaminetetraacetic acid

EFA	Essential fatty acid
ELISA	Enzyme-linked immunosorbent assay
EPA	Eicosapentaenoic acid
F2-iso	8-iso-PGF2α
FABP	Fatty acid-binding protein
FFA	Free fatty acid
FFQ	Food frequency questionnaire
GLA	γ-linolenic acid
HDL	High density lipoprotein
HPLC	High performance liquid chromatography
hs-CRP	High-sensitivity C-reactive protein
IBRS	Inflammatory biomarker risk score
IL-6	Interleukin-6
IS	Ischemic stoke
JNK	c-jun-NH2-terminal kinase
LA	linoleic acid
LBM	Lean body mass
LC	Long chain
LDL	Low density lipoprotein
LPL	Lipoprotein lipase
MAPK	Mitogen-activated protein kinase
MI	Myocardinal infarction

MUFA	Monounsaturated fatty acid
NF-κB	Nuclear factor-κB
Ox-LDL	Oxidized low density lipoprotein
PGF	Prostaglandin
PL	Phospholipids
PPAR	Peroxisome proliferator activated receptor
Pro	Proline
PUFA	Polyunsaturated fatty acid
RCT	Randomized clinical trial
ROS	Reactive oxygen species
SCD-1	Stearoyl-CoA desaturase-1
SFA	Saturated fatty acid
SREBP-1	Sterol regulatory element binding protein-1
T2D	Type 2 diabetes
TCA	Tricarboxylic acid cycle (also known as citric acid cycle)
TG	Triglycerides
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-α
VIIIc	Factor VIII
VLDL	Very low density lipoprotein
vWF	von Willebrand factor
WBC	White blood cell count

WHR	Waist-to-hip ratio

PREFACE

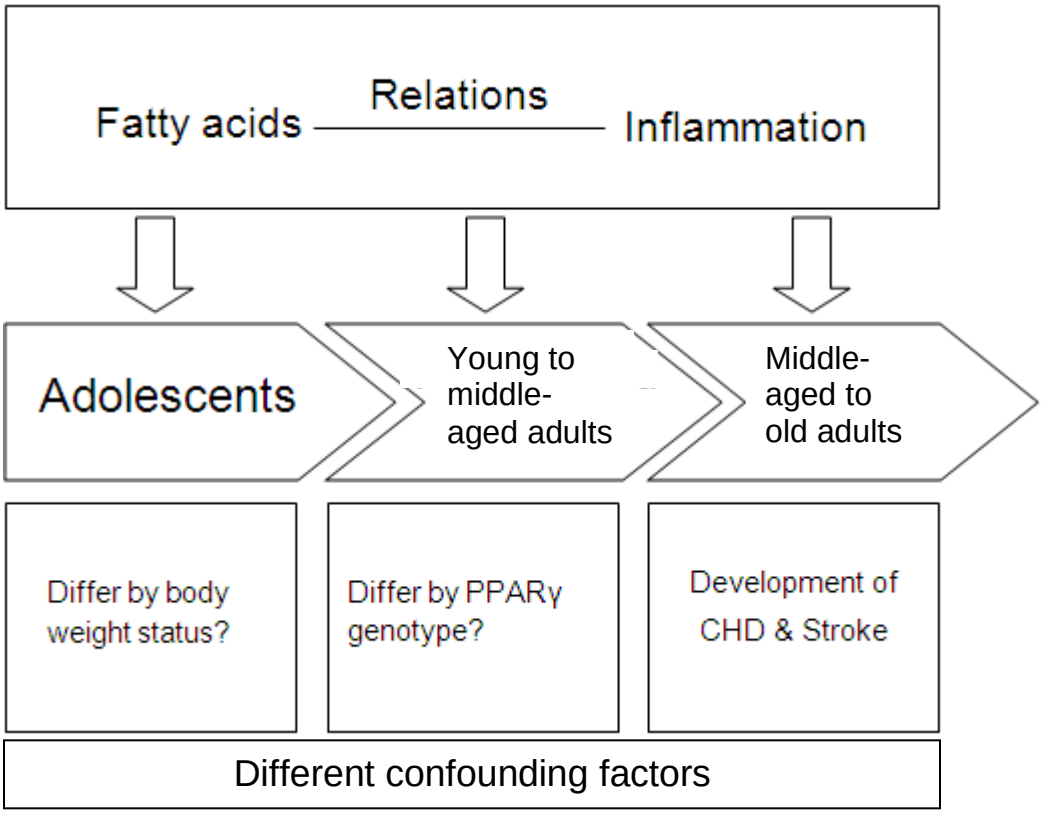
Dietary fatty acid intake, reflected by the endogenous fatty acid profile, has been associated with cardiovascular diseases (CVD) risk. Additional evidence is needed regarding the various health effects of individual fatty acids in the pathogenesis of inflammation, an important contributor to the development of CVD. Since CVD risk may emerge in childhood, this dissertation aims to explore and help better understand the relations between fatty acids, inflammation and CVD health among different age groups (i.e. adolescents, young adults, middle-aged adults).

An extensive literature review was conducted to determine the existing scientific evidence on the relation between fatty acid metabolism and inflammation pathogenesis, as well as how they were interrelated with cardiovascular health across the lifecycle. The details of the four studies, including study populations and data collection methods were then described. Manuscript 1 and its supplemental analyses examined the cross-sectional associations between fatty acids (as measured in serum phospholipids) and inflammation among adolescents (mean age 15 years), and whether adiposity modified these associations. Manuscript 2 discussed the modifying effect of the genetic variant, peroxisome proliferator-activated receptor gamma [PPARγ] Pro12Ala polymorphism, on the relations of dietary polyunsaturated fatty acid intake with inflammation among young adults. Additional analyses also examined the same study question for the intakes of saturated and monounsatured fatty acids. The prospective data from the Atherosclerosis Risk in Communities (ARIC) study of middle-aged adults was used in both Manuscripts 3 and 4. Specifically, Manuscript 3 examined the modification effect of dietary fatty acid

intake on the associations between inflammation and CVD outcomes (i.e. coronary heart disease [CHD] and ischemic stroke [IS]), while Manuscript 4 explored the mediation effect of inflammation on the relations between plasma phospholipid fatty acids with CHD and IS.

The following diagram illustrates the general scheme of this dissertation.

DISSERTATION SCHEME



CHAPTER 1. INTRODUCTION

1.1 Fatty acids and types of fatty acids

Fatty acids are fundamental components of natural fats and oils. Biochemically, they are carboxylic acids with at least four carbon atoms (with or without branch chains). Fatty acids can be grouped as saturated (SFA), monounsaturated (MUFA) or polyunsaturated fatty acids (PUFA) depending on how many double bonds they have in the chain (**Table 1.1**, List of common fatty acids, page 29). According to the position of double bonds, unsaturated fatty acids can also be grouped as ω9, ω6 or ω3 family. In most mammals, fatty acids are synthesized from acetyl-CoA (two carbons), for which glucose is the primary source. Due to the nature of their biosynthesis, natural fatty acids usually exist with even numbers of carbons and cis-configuration. However, among bacteria and lower plants or animals, fatty acids with odd-numbered carbon chains, such as pentadecanoic acid (15:0), heptadecanoic acid (17:0), etc, are also frequently seen[1]. These odd-numbered carbon chain fatty acids can either be de novo synthesized from propanyl-CoA or produced from the α-oxidation of even-numbered carbon chain fatty acids[2].

1.2 Fatty acid metabolism

1.2.1 Anabolism

In the human body, the liver is the primary site for fatty acid synthesis. Palmitic acid (16:0) is the first fatty acid generated via *de novo* lipogenesis, from which longer fatty acids or unsaturated fatty acids can be formed by elongation and desaturation reactions. Although we have the capability to synthesize the ω9 family using Δ^9 desaturase, we

cannot produce ω3 and ω6 PUFAs, unless obtaining essential fatty acids (EFA), i.e. linoleic acid (LA, 18:2 ω6) and α-linolenic acid (ALA, 18:3 ω3), from dietary sources (e.g. plant oils). Humans are able to convert ALA and LA into longer ω3 and ω6 PUFAs, respectively, including eicosapentaenoic acid (EPA, 20:5 ω3), docosahexaenoic acid (DHA, 22:6 ω3), and arachidonic acid (AA, 20:4 ω6), etc. However, it has been shown in metabolic studies that such conversion is fairly limited, with an approximate rate of <8% for EPA and < 0.1% for DHA among adult men, but slightly higher among adult women[3-5]. In addition, since only one set of elongase and desaturase (including Δ^9, Δ^6, Δ^5 and Δ^4 desaturases in human body) is shared by both of ω3 and ω6 PUFA, two conversion pathways are competitive with each other. And during the EFA deficiency, the ω9 PUFA will accumulate, especially the eicosatrienoic acid (20:3 ω9). The biosynthesis of some commonly seen fatty acids in the human body is illustrated in **Figure 1.1** (page 30).

1.2.2 Catabolism

In the human body, fatty acids can be "free fatty acids" (FFA) or be esterified to form triglycerides (TG, each molecule is made up of a glycerol and three fatty acids), phospholipids (PL), cholesterol esters (CE) or other esterified compounds. However, since FFAs are water insoluble, especially long-chain (LC) fatty acids, they must be transported by albumin in blood. After being taken up by tissues and activated, FFAs can be catabolized. Different from fatty acid synthesis, which takes place in the cytosol, fatty acid oxidation (i.e. β-oxidation) in the mitochondria requires the presence of oxygen. A large amount of energy (i.e. ATP) is produced from the breakdown of fatty acids, while the following two pathways are involved. One is via the respiratory (electron-transfer)

chain; and the other is via the generation of acetyl-CoA. In terms of the second pathway, oxidation of an odd-numbered carbon chain fatty acid yields propionyl-CoA instead of acetyl-CoA, while both products can then enter the tricarboxylic acid (TCA) cycle (also named as citric acid cycle). However, when fatty acids exceed the capacity of the liver for fatty acid oxidation, ketone bodies (e.g. acetoacetate) will be generated and utilized by extrahepatic tissues (e.g. muscle) as energy sources. **Figure 1.2** (page 31) shows the general reactions of fatty acid metabolism. Compared to protein or carbohydrate catabolism, fatty acid oxidation provides more energy, with an estimation of 9 kcal/gram of fat versus 4 kcal/gram of protein or carbohydrate.

Fatty acid containing lipids-are important storage and functional forms of fatty acids in the human body. For example, PL is a major component of cell membranes. Nevertheless, all lipid droplets (primarily TG and cholesterols) are hydrophobic and must be transported from the site of absorption/synthesis/storage (i.e. intestine/liver/adipose tissue) to tissues of utilization, for which lipoproteins are good vectors. A lipoprotein consists of TG, free (unesterified) cholesterol, CE, PL monolayer and protein components, i.e. apolipoproteins. There are four kinds of lipoproteins in our body, including chylomicrons, very low-density lipoproteins (VLDL), low-density lipoproteins (LDL, the product of VLDL metabolism) and high-density lipoproteins (HDL). Specifically, chylomicrons are responsible for transporting dietary (exogenous) TG from intestine to peripheral tissues during fat absorption; VLDL and LDL transports endogenous TG and cholesterol from liver to the rest of the body; whereas HDL sequesters excess cholesterol from tissues and blood lipoproteins and transfers

apolipoproteins between other lipoproteins. Apolipoproteins plays a critical role during these metabolic processes, which can serve as enzyme (e.g. lipase) cofactors and as ligands for interaction with lipoprotein receptors. When taken up by peripheral tissues via lipoprotein lipase (LPL), for example, lipoproteins can release FFAs for utilization. **Figure 1.3** (page 32) depicts the details of lipid transportation and utilization.

1.2.3 Regulation

The nutritional status of the body is the fundamental factor influencing fatty acid metabolism, with two hormones originating in the pancreas, i.e. insulin and glucagon, as primary regulators. Changes of fatty acid metabolism in different tissues during normal feeding and postprandial (or fasting) phases are presented in **Table 1.2** (page 33). Meanwhile, at the molecular level, there are many genes and their products participating in the regulation, under the control of insulin and glucagon. For example, sterol regulatory element binding protein (SREBP-1) and carbohydrate responsive element binding protein (ChREBP) can promote fatty acid synthesis. Elevated insulin level increases the activity of SREBP-1, while ChREBP is activated by glucose. And in response to the low-energy status, the activity of AMP-activated protein kinase (AMPK) can be stimulated, which then favors fatty acid oxidation, but inhibits fatty acid synthesis. In addition, peroxisome proliferator activated receptors (PPARs) is another important group of genetic regulation factors of fatty acid metabolism, the details of their functions are described in **Section 1.5**.

1.2.4 Dietary fatty acid intakes and their biomarkers

As an important macronutrient, fatty acids have been associated with the prognosis of diseases, as shown in numerous epidemiologic studies. Dietary fatty acid intakes are commonly assessed using food frequency questionnaires (FFQ). However, this self-administered instrument has several limitations. For example, the questions may be too vague to record intake of individual foods; queries about the actual quantity and type of food in FFQs are limited, while many details, such as food preparation and additions, are usually not collected; self-reporting bias may occur which may impair the accuracy and reliability ofdata collected[6]. Therefore, identification of potential biomarkers which may be used as objective measurements of dietary fatty acid intake, may be valid and quantifiable predictors of diseases.

It has been shown that the composition and level of blood (serum or plasma) PL fatty acids may well reflect both short-term (2-6 weeks) and long-term (several months or years) dietary fatty acid intakes[7-11]. Improvements in gas chromatography and high performance liquid chromatography (HPLC) methods have improved the feasibility of measuring individual fatty acids and their isomers in large epidemiologic studies[12]. It is important to keep in mind that SFAs and MUFAs may be *de novo* synthesized from carbohydrates, and thus endogenous levels may not provide a good measure of dietary SFA and MUFA intakes; whereas, blood levels of PUFAs, which cannot be synthesized endogenously without consuming dietary LA and ALA, are generally considered better markers for dietary intake[12]. In addition to dietary intake, other factors influence fatty acid composition including gender, smoking, physical activity, weight status and diabetes status for example.

1.3 Fatty acids, inflammation, and cardiovascular disease

1.3.1 Atherosclerosis, inflammation, and CVD risk

Atherosclerosis is a common vascular pathological condition, in which the wall of arteries thickens due to the deposition of circulating fat components. Atherosclerosis is the primary cause of and commonly equated to coronary heart disease (CHD), and it is also associated with stroke, heart failure, and other conditions.

Fatty acids have been widely associated with the development of cardiovascular disease (CVD) and its risk factors. The fat components of LDL, for example, are oxidized resulting in oxidized-LDL (Ox-LDL), which then damage the endothelial cells of vascular smooth muscle and result in endothelial dysfunction. However, the lesions can also be caused by virus or bacterial infection, toxins (e.g. smoking), etc. In response to the injury, endothelial cells express several kinds of cell signaling molecules, such as selective adhesion molecules, to recruit immune cells (i.e. neutrophils, macrophages, etc.) to the injured sites and initiate the inflammatory process. Pro-inflammatory cytokines can then be secreted by immune cells, such as C-reactive protein (CRP), inflammatory cytokines interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α), to uptake the oxidized fat components and further recruit more immune cells. In this respect, inflammatory reaction is originally an immunological attempt of vascular tissues to protect themselves from either endogenous or exogenous harmful stimuli, and well regulated by our body (as described in **Figure 1.4**, page 34).

Prolonged or chronic inflammation can have detrimental effects, because during this process, reactive oxygen species (ROS) are generated by macrophages, further oxidize

Ox-LDL, which are, in turn, taken up by macrophages. As a result, macrophages change into foam cells, accumulate and finally plaques are formed to thicken the arterial wall. At the same time, pro-inflammatory cytokines can stimulate the vascular smooth muscle cells to multiply, migrate to and cover the surface of the plaques. Essentially the plaques become fibrous, make the inner-diameter of arteries narrower and allow less blood flow. What is worse, those plaques are fragile. When they rupture, arteries can be easily blocked and the blood supply may be completely cut off. In such cases, thrombosis happens. It is important to note that the overproduction of ROS is a key point here. Under normal conditions, our body has an antioxidant system to scavenge oxidants. Adequate amount of ROS is beneficial because of its role in attacking and killing pathogens, as well as participating in cell signaling pathways. However, under circumstances of inflammation, the amount of oxidants overwhelms the capacity of the antioxidant system, which subsequently creates oxidative stress. Oxidative stress has tremendously toxic effects on cells, which damages proteins, nucleotides, lipids, etc. and is associated with many diseases as well as CVD risk factors, such as obesity, hypertension, metabolic syndromes, diabetes, etc[13].

1.3.2 Biomarkers of inflammation process

Inflammation, hemostatic dysfunction and oxidative stress are closely intertwined and play critical roles in the development of CVD[14, 15], thus, detecting and reducing the progression of inflammation may prevent the development of CVD[16]. A number of biomarkers for these pathological conditions have been identified and are used widely in studies among various populations.

CRP (especially high-sensitivity CRP, hs-CRP), a nonspecific acute-phase reactant, has been widely used to predict CVD, because of its easy and reliable measurement[17]. Likewise, white blood cell (i.e. leukocytes) count is also an indicator for inflammation[18, 19]. Cytokines, such as IL-6 and TNF-α, are released by immune cells during inflammation and oxidative stress[20]. TNF-α may activate NF-κB, mediating the expression of a variety of proteins (e.g. IL-6) for an inflammatory response, while IL-6 is also found to have anti-inflammatory effects by stimulating the production of anti-inflammation cytokines and suppressing TNF-α[21-23]. In addition, 15-keto-dihydro-PGF2α, a major metabolite of the prostaglandin F2α (PGF2α), is formed at the site of inflammation, and may indicate in vivo cyclooxygenase-mediated inflammation[24].

Another common inflammatory biomarker is fibrinogen (a plasma glycoprotein), which is involved in the coagulation cascade during convertion into fibrin, could increase its level with tissue inflammation or tissue destruction[25]. The excessive generation of fibrin from fibrinogen in the vascular system may lead to thrombosis[25]. Notably, in addition to fibrinogen, a few other factors, such as von Willebrand factor (vWF) and VIII factor, are also involved in and essential for activating the coagulation cascade in response to the vascular tissue damage[26].

F2-isoprostanes (the most frequently studied one is 8-iso-PGF2α), derived from the oxidation of arachidonic acid (AA, 20:4ω6) as a free radical-mediated lipid peroxidation product has been shown to be an effective biomarker of oxidative stress[27, 28]. To the contrary, elevated blood level of adiponectin, the most abundant protein in adipocytes, is associated with reduced CVD risk in almost all studies which may be through increasing

tissue fat oxidation and inhibiting the expression of adhesion molecules in vascular endothelial cells and cytokine production from macrophages[29]. Notably, the cardioprotection of adiponectin, as well as the pro-inflammatory and pro-oxidative effects of IL-6, TNF-α, CRP, 15-keto-dihydro-PGF2α and 8-iso-PGF2α have also been noted in young population, including children[30-33].

Serum albumin is the most abundant transport protein for fatty acids, which is synthesized in the liver and can be down-regulated under the conditions of inflammation and malnutrition[34, 35]. In a few studies, low level, versus relatively high level, of serum albumin has been found to be related to higher risk of CVD risk[36-38].

1.3.3 Role of fatty acids in CVD risk

There have been numerous studies showing significant relations between dietary or plasma fatty acids and CVD risk factors[39, 40], including inflammation[41, 42]. Both the amount and type of fatty acids contribute to the development of inflammation. For example, increased intake of dietary fats may result in greater amount of circulating chylomicron, triglycerides and LDL postprandially[43-46], which has been shown in clinical trials[45, 46]. Prolonged exposure of these fat components to oxidation may overwhelm the control capability of the immune system. Under such circumstance, as mentioned in section 1.3.1, the inflammatory process is likely to increase.

Type of fatty acid influences the rate of utilization. Dietary and blood SFAs are utilized slower (i.e. slower β-oxidation) than unsaturated fatty acids[47], which may lead to higher accumulation of circulating SFAs and generally considered deleterious to the cardiovascular system[40]. SFAs have been found to increase levels of inflammation,

oxidation, and adverse CVD risk factors, whereas MUFAs and PUFAs are usually thought to be beneficial[40, 48]. In a meta-analysis of 60 clinical trials, the ratio of total to HDL cholesterol was reduced by replacing dietary SFAs with unsaturated fatty acids[49]. Further, in a prospective nested case-control study in the Physicians' Health Study with up to 17-years follow-up, levels of blood ω3 PUFAs were shown to be inversely related to the risk of sudden death[50]. Complex signaling pathways are involved in the functions of SFAs, with the toll-like receptor (TLR) as an important mediator[51]. If TLR is mutated or absent, the adverse effects of SFAs may be attenuated or eliminated[52, 53]. It has been widely reported that TLR may be activated by SFAs in murine adipocytes and macrophages, which then results in the activations of several signaling pathways, such as nuclear factor-κB (NF-κB), c-jun-NH_2-terminal kinase (JNK), mitogen-activated protein kinase (MAPK), and the productions of cytokines[54-56].

The potential cardioprotective mechanisms for ω3 PUFAs include exertion of antiarrhythmic effects, decreased platelet aggregation, lower plasma triglycerides, higher HDL-cholesterol and LDL particle size, lower blood pressure, reversed cholesterol accumulation, and decreasing inflammation process[57-59]. **Figure 1.5** (page 35) summarizes some key roles of ω3 PUFAs and its anti-inflammatory properties. In addition, the newly discovered resolvins and protectins, which are derivatives of EPA and DHA, could also actively participate in the inflammation resolving[60].

The evidence is inconsistent regarding the protective effects of unsaturated fatty acids due to several potential reasons. For example, Balk et al. conducted a systematic review of randomized clinical trials (RCT) to evaluate the effect of consumption of ω3 PUFAs

on serum CVD risk factors. Although this study supported the beneficial effects of fish oil on serum lipid levels, no conclusive evidence demonstrated an inverse association between ω3 PUFAs (e.g. ALA) and high-sensitivity CRP (hs-CRP) level[61]. The effects of ω6 PUFAs are also widely debated. The primary argument focuses on the oxidation of unsaturated FAs, which may create inflammatory responses and oxidative stress[62, 63]. Specifically, the more double bonds a fatty acid has, the greater probability to promote lipid peroxidation[64], thus exponentially increasing lipid peroxyl radicals, one kind of ROS (as shown in **Figure 1.6**, page 36). Excessive amount of lipid peroxyl radicals are extremely toxic to cells by reacting with other lipids, causing DNA mutations, cross-linking proteins, which essentially destroy the integrity and functions of cell membranes, as well as other cell activities. Such adverse effects are of particular concern during supplementation of unsaturated fatty acids, which may explain the differences in results among studies on the effects of ω3 PUFAs in primary and secondary prevention. However, the effects of damage from the oxidation of ω3 PUFAs has not been well explored in human subjects, neither consistently confirmed by experimental studies, and further study is warranted.

Although unsaturated fatty acids may promote inflammatory/oxidative responses to some degree, SFAs present much more robust harmful effects on CVD risk factors. For instance, in an experimental study on murine adipocytes, palmitic acid (16:0) significantly increased the production of TNF-α and IL-10. Treating cells with DHA (22:6 ω3) had no impact on TNF-α level, and oleic acid (18:1 ω9) treatment also resulted in no change of either cytokine level[65]. Human studies reported that consumption of a

SFA-rich diet can result in higher serum/plasma LDL concentration, compared to the MUFA or PUFA-rich diets[66-68]; while the VLDL secretion could be suppressed by hepatocytes incubated with chylomicron remnants enriched in ω3 PUFAs[69]. Therefore, based on a large pool of evidence, the Dietary Guidelines Advisory Committee and the American Dietetic Association recommended intakes of unsaturated fatty acids (i.e. MUFAs, ω3 PUFAs and ω6 PUFAs), and despite some differences in quantity of intake; reduced consumption of SFAs is suggested[70, 71].

Although the effects of different types of fatty acids on CHD have been relatively well established, evidence about the associations of fatty acids with ischemic stroke (IS) is inconsistent, due to lack of well-designed epidemiological studies[72]. Several research groups have reported that the established associations between types of fatty acids and CHD did not consistently apply to IS and its risk factors[72-74]. For example, increased SFA intake was associated with reduced risk of IS in males enrolled in the Framingham Heart Study[75]. And Sanders et al. did not observe any influence of varying the ratio of dietary ω6/ω3 fatty acids on IS-related hemostatic factors[76]. Considering that IS shares similar pathological mechanisms with CHD, further study of the fatty acid/IS relationship is warranted.

1.3.4 Diverse roles of individual fatty acids in inflammation and oxidative stress

Since fatty acids in natural foods exist as complexes and mixtures, it is of interest to study fatty acids in classes, namely as SFAs, MUFAs and PUFAs. Nonetheless, the role of individual fatty acids in fatty acid-CVD risk relations should be examined as well. Studying individual fatty acids may facilitate not only the understanding of complicated

fatty acid-CVD risk relations, but may also clarify the specific functions of a single fatty acid and is useful for advising food manufacturers. Although there have been an increasing number of studies conducted to examine the health effects of diverse individual fatty acids, findings are inconsistent. Therefore, additional study is necessary to elucidate the relations between individual fatty acids and inflammation.

1.3.4.1 Saturated fatty acids

Palmitic acid (16:0), the major fat component of animal products, is generally found to exert pro-inflammatory and pro-oxidative effects[40, 77]. According to a recent in vitro study, nicotinamide adenine dinucleotides phosphate (NADPH) oxidase-dependent superoxide could be produced during the stimulation of TLR4 by palmitate, which then activates the inflammatory factor NF-κB in endothelial cells[78]. Consistently, Fernandez-Real et al. observed among 116 healthy participants that the plasma proportion of palmitic acid was significantly correlated with lower adiponectin level, especially among women and nonsmokers[79].

Stearic acid (18:0), a fatty acid found mainly in animal products, makes up part of the composition of several kinds of vegetable oils, such as soybean oil. Different from palmitic acid, however, stearic acid has been shown to provide certain beneficial effects on CVD risk factors. For instance, results of a case-control study showed that the percentage of serum free stearic acid was inversely associated with the odds of myocardial infarction (MI)[80]. And recently, a systematic review demonstrated that compared with other SFAs, stearic acid lowered the LDL level and the ratio of total to HDL cholesterol. However, the authors also claimed that the level of fibrinogen might be

increased by high intake of stearic acid[81]. Therefore, the role of stearic acid on CVD risk deserves further examination. Compared to palmitic acid, about 30~40% less stearic acid was incorporated into triglyceride and cholesterol ester, but, instead, about 40% more stearic acid was incorporated into phosphatidylcholine[47]. Additionally, the desaturation of stearic acid to oleic acid was 2.4 times higher than that of palmitic acid to palmitoleic acid[47], This evidence may be a possible explanation for less 'unhealthy effects' of stearic acid. The blood levels of some SFAs, such as myristic acid (14:0), pentadecanoic acid (15:0) and heptadecanoic acid (17:0), mirror dairy product intake[82-84]. A few previous studies have shown favorable associations between these dairy SFAs with CVD event incidence and CVD risk, e.g. BMI, blood lipids level, insulin level, etc[82, 84-87], including a study among healthy adolescents[88]. In that study, serum myristic acid was inversely related to LDL levels[88]. Nevertheless, findings are controversial; while research regarding the effects of these three individual fatty acids on inflammation and oxidative stress are not well understood. In contrast to Sun et al., who reported that women with higher plasma concentrations of pentadecanoic acid had a significantly greater risk of ischemic heart disease (IS)[87], Warensjo et al. [89] found an inverse relation between serum pentadecanoic acid and heptadecanoic acid with first-ever acute myocardial infarction. There was one blinded, randomized, crossover study demonstrating the substantial suppression of oxidative stress and inflammation with greater dairy food intake among overweight and obese adult subjects[85]. However, such results cannot be extended to individual SFAs, since dairy foods represent a whole food group containing several individual fatty acids, vitamins, minerals and other food compounds as well.

1.3.4.2 Monounsaturated fatty acids

The two prominent MUFAs are palmitoleic acid (16:1) and oleic acid (18:1), both of which can be synthesized in our body from SFAs. Oleic acid may also be directly obtained from dietary MUFAs, such as olive oil, the main source of fat in the Mediterranean diet; while blood palmitoleic acid has very limited dietary sources and thus dependent on the conversion of endogenous palmitic acid[90]. The majority of evidence, thus far, identifies palmitoleic acid as harmful, showing adverse effects of endogenous palmitoleic acid on lipoprotein profiles, aortic cholesterol accumulation, triglyceridemia, abdominal adiposity, fasting insulin, CRP level[90-93] and endothelia-dependent vasodilation[94]. Interestingly, oleic acid may play a more "neutral role" in the progression of CVD risk[92], compared to palmitoleic acid, as it is commonly believed that oleic acid is cardioprotective[95].

From the perspective of inflammation and oxidative stress, Petersson et al. observed a positive relation between serum oleic acid and CRP over 20 years of follow-up among middle-aged subjects[93], as well as in a cross-sectional study of 264 Swedish elderly[91]. In a multi-center crossover RCT among 200 healthy adults, the elevated oleic acid content of LDL was associated with lower F2-isoprostane levels after 13 weeks of intervention, but not between the study intervals[96]. Inconsistent results are often demonstrated between intervention and observational studies, and therefore further exploration is necessary.

1.3.4.3 Polyunsaturated fatty acids

Endogenous PUFAs were generally found to reflect dietary intakes relatively well. According to a previous study among 3570 middle-aged adults, the Pearson correlation

(r) between dietary and plasma total PUFAs (expressed as % of total FAs) for PL and CE was 0.25 and 0.31, respectively[9]. Both of PL and CE DHA were moderately correlated with dietary DHA (r=0.42); while for LA, ALA and EPA, r was lower, ranging from 0.15 to 0.28[9]. And in some other studies, even higher correlations were reported[12].

When examining the effects of individual PUFAs on health or diseases, many studies tend to use mixtures of several PUFAs, such as fish oil (containing EPA and DHA, or further combined with LA or ALA). For example, in a cross-sectional study among 992 patients with stable CHD, the level of LC ω-3 PUFA (EPA+DHA) was inversely associated with CRP and IL-6[97]. In a double-blind, controlled intervention, IL-6 concentration decreased with fish oil administration over 8-weeks, while such a reduction did not vary by LA intake[98]. Intervention using food mixtures (e.g. fish oil), instead of single nutrients(e.g. EPA or DHA) may facilitate the investigation of the synergistic effects of nutrients. Questions have been raised about the high ratio of ω-3/ω-6 PUFAs, rather than the high absolute amount of ω-3 PUFAs, that contribute to cardioprotection[99].

However, the health effects of individual PUFAs should be addressed as well, since the function of each PUFA can be different from the others. Such diversity exists between EPA and DHA. In animal and *in vitro* studies, some reported that plasma EPA might be more efficient in reducing inflammatory responses than DHA[100, 101], while others showed the opposite[102]. Similar inconsistensies have been shown in population studies[103-105]. In a double-blinded, placebo-controlled study, the level of sICAM-1 (an inflammatory marker) had stronger association with plasma DHA than with EPA, despite lower dosage of DHA administration[104]. This result indicated that DHA might have a

greater potential anti-inflammatory effect[104]. In contrast, in a 6-week RCT among patients with T2D, neither purified EPA nor DHA substantially reduced IL-6 or CRP levels, while the level of TNF-α was significantly decreased by both LC ω-3 PUFAs[105]. Considering the metabolic differences of LC ω-3 PUFAs in the body, future research is necessary to sort out these differing results.

It is well known that flaxseed oil is rich in ALA, and by consuming flaxseed, the serum level of ALA may be significantly increased[106]. ALA is an essential ω-3 PUFA that generates longer ω-3 PUFAs in the body, suggesting its metabolic importance even though it may not be as efficient as EPA or DHA in preventing CVD risk[107]. In a recent cross-sectional study, dietary ALA consumption was reported to be inversely associated with CRP level among a group of Japanese[108]. However, inconsistent results have been demonstrated for the effects of ALA on inflammatory markers in RCTs. In one 4-week clinical study, consumption of flaxseed oil-based diet (containing approximate 14g/d ALA) lowered levels of TNF-α and IL-1 by about 30% in healthy participants[109]. In a 12-week intervention, a similar amount of ALA from flaxseed oil reduced IL-6 levels in older men only but not women[110]. In contrast, some RCTs with lower dosages of ALA failed to demonstrate any changes in inflammatory markers[111-113]. To explain these differences, Calder proposed that increasing the ALA intake to >10g/d may be required to exert anti-inflammatory benefits[107]; while Mozaffarian stated that 2~3g/d ALA may be sufficient to prevent CVD risk based on the evidence reviewed[114].

In 2009, several nutritional and epidemiologic expert committees, supported by AHA, suggested that "the consumption of at least 5% to 10% of energy from ω-6 PUFAs

reduces the risk of CHD relative to lower intakes"[115]. This statement was primarily based on evidence about LA, since it is an essential FA accounting for 85% to 90% of the dietary ω6 PUFAs and responsible for the generation of longer ω6 PUFAs, such as AA, in our body[115]. For this reason, however, arguments are also raised, since AA, the substrate for many eicosanoids (**Figure 1.7**, page 37), has been widely linked to various aspects of pathological immunity and inflammation processes[116]. Eicosanoids are a group of derivatives from PUFAs, which include prostaglandins, prostacydins, thromboxanes and leukotrienes. Although some of these may also be derived from LC ω3 PUFAs (i.e. EPA and DHA) and exhibit anti-inflammatory effects, the AA-derived eicosanoids (e.g. prostaglandin E_2, thromboxane A_2, leukotriene B_4, etc.) are generally pro-inflammatory[116]. Therefore, LA, a FA primarily from plant sources, may have certain favorable effects on CVD risk; however as the metabolic precursor of AA, increasing its level may lead to inflammation and high CVD risk[115]. It has also been proposed that the increased proportion of LA (in diet and thus in tissue) may reduce the generation of endogenous LC ω3 PUFAs[117]. And one possible mechanism may be due to the competition between ALA and LA for the same desaturation and elongation enzymes during the conversions to longer PUFAs[118],

Notably, there is limited capability to convert AA endogenously from dietary LA, which, according to previous results of a 12-week dietary intervention, may be as low as about 0.2%[119]. Therefore, it is not surprising that although the effects of LA on lowering some traditional CVD risk factors, i.e. cholesterol level, are relatively well-established[115], controversies exist. There is little evidence specifically targeting the LA-

inflammation/oxidative stress relationship. Previously, *in vitro* studies have shown that LA might induce endothelial cell activation and vascular inflammation via both PI3K/Akt- and ERK1/2-mediated proinflammatory signaling pathways[120]. And vascular smooth muscle cells could increase the production of inflammatory chemokine (i.e. IL-8) in response to LA stimulus under the condition of oxidative stress, which was mediated by AA metabolites[121]. In contrast, in a human study conducted among a Japanese group found that increased intake of LA was significantly associated with lower CRP level in men[108]. Damsgaard et al. did not observe any effects of an 8-week LA administration on the production of TNF-α, IL-10 or interferon-γ in the peripheral blood mononuclear cells of healthy men[98]. Accordingly, in the American Heart Association (AHA) advisory paper, the experts concluded that a net proinflammatory effect of LA is hardly supported in humans[115].

There are similar debates about the inflammatory effects of AA, as previously mentioned. Theoretically, AA tends to promote the inflammatory processes and oxidative stress[116], which has already been shown in many *in vitro* or animal studies. Nevertheless, human studies appear to support the anti-inflammatory effects of AA to some extent, despite differences of study designs, populations, dosages, etc. For example, in a cross-sectional study among 1123 participants, Ferrucci et al. observed inverse associations between plasma levels of AA and IL-6, as well as IL-1ra (another acute-phase protein)[122]. Likewise, another cross-sectional study conducted by Pischon et al. concluded that ω6 PUFAs might enhance, rather than inhibiting the anti-inflammatory effects of ω3 PUFAs[123]. And in line with these observational findings, serum proportions of AA

increased after a 12-week very-low-carbohydrate dietary intervention, which was significantly related to the decrease of several biomarkers of inflammation and endothelial dysfuncition, i.e. TNF-α, IL-6, IL-8, MCP-1, E-selectin, I-CAM, and PAI-1[124].

Compared to LA and AA, other ω6 PUFAs, e.g. γ-linolenic acid (GLA, 18:3 ω6) and dihomo-γ-linolenic acid (DGLA) are less well investigated. Green leafy vegetables and nuts are primary sources of GLA in nature, although GLA exists in trace amounts[125]. In addition to dietary GLA sources, GLA can be produced from dietary LA in the human body. There has been evidence showing GLA as an anti-inflammatory fatty acid[125]. The potential mechanisms for the beneficial effects of GLA may be through its metabolites, DGLA, which can further generate a variety of anti-inflammatory eicosanoids[125].

1.4 Lifecycle nutrition and cardiovascular health

Fatty acids are important macronutrients that represent a group of modifiable factors for CVD risk prevention over the lifecycle, including inflammation process. Therefore, it is important to study and understand better the relationship of fatty acids with markers of inflammation on CVD risk, especially among different age groups. However, there are many influencing or confounding factors (either endogenous or environmental) that should be considered in elucidating these relations.

It is well understood that CVD is an age-related chronic disease and chronological age is a common measurement of aging. With aging per se, there are structural and functional changes in the heart[126] (summarized in **Table 1.3**, page 38). Compared to young populations, elderly adults may have a blood vessel wall thickened with pre-deposited

collagen. Therefore, older people may be more susceptible to the oxidized fat components and accentuated pathogenesis of atherosclerosis. Especially, oxidation products or other metabolic substances may accumulate to harmful levels in the body overtime. For example, about 30% of the original Framingham Study population (aged 67-96 years) was observed with high blood concentrations of homocysteine[127]; and in the Scottish Heart Health Study, plasma fibrinogen was found to increase with age[128]. Both homocysteine and fibrinogen are believed to be associated with higher CVD risk at excessive levels[129, 130]. Meanwhile, the metabolism in the body may change as well during the aging process. It was reported that male participants who were aged 20+ years metabolized dietary DHA much faster than their counterparts aged 60+ years[131]. Such age-related changes may also be related to gender. Women tend to suffer from CVD in older ages, compared to men[132] and estrogen levels may explain the favorable CVD risk factor profiles of women[133].

In addition, it is known that sustained inflammation may be due to the dysfunction of our immune system under pathological conditions; whereas changes of immunity are also age-related[134]. We generally have a stronger and more active immune system as children, while immune functions decline with aging as seen in elderly adults. For example, the plasma level of TNF-α was found to be higher in elderly than young, healthy subjects[135], indicating more inflammation in the former group.

Environmental factors (e.g. lifestyle including diet, smoking, etc)[126] may differ by age. For example, the rates of smoking, an important CVD risk factor, are generally lower among older populations. In a prospective cohort study of 14786 Finnish adults,

compared to participants aged 25-49 years, 10.5% fewer of men and 16.7% fewer of women aged 60-64 years were smokers[136] (**Table 1.4**, page 39). Such differences in environmental factors may significantly impact the development of inflammation and oxidative stress and other CVD risk (e.g. hypertension, adverse endogenous lipid profiles, obesity, diabetes[137-140]). Even though elderly adults smoke less, they may still have worse CVD risk factor profiles than young population (**Table 1.4,** page 39). Age-related changes in the body, as mentioned previously, may explain this decline. Further study of the relations between fatty acids, inflammation, with CVD risk in middle-aged adults is warranted, taking into account all of these potential confounding factors.

1.5 Influence of genetic factors

Generally, genetic factors influence the relationships among fatty acids, inflammation and CVD risk in two ways. First, aging is linked to these relations as mentioned previously (see section 1.4). Second, genetic variation among individuals and populations influences cardiovascular function. Certain genotypes may slow the progression or degeneration of physiological systems by responding differently to environmental exposures (e.g. diet[141, 142]). For example, limiting dietary SFA intake may be particularly important for preventing or lowering CVD risk among carriers of the Ala allele of the fatty acid-binding protein 2 (FABP2)[143].

Among many genetic regulation factors of fatty acid metabolism, peroxisome proliferator activated receptors (PPARs) is an important group, including three members, α, β, and γ, which are distributed primarily in liver, muscle and adipose tissues, respectively[144]. PPARα and PPARβ are involved mostly in β-oxidation, while PPARγ

regulates fatty acid storage[144], promotes lipid uptake and adipogenesis by adipocytes[145]. Both natural fatty acids and synthetic drugs can be the ligands for PPARγ, which stimulate PPARγ to form heterodimers with retinoic X receptors (RXR), bind to PPAR response elements (PPREs), and control various downstream genes[146] (**Figure 1.8**, page 40). PPARγ is believed to be atheroprotective, on one hand, via modulating the expression of anti-inflammatory genes[147]. These genes could be involved in inhibiting the inflammation reactions of immune cells and preventing possible harmful effects of fat accumulation in tissues (e.g. blood vessels, liver and skeletal muscle), etc[147]. On the other hand, PPARγ may also act in a ligand-dependent manner to repress the inflammatory gene expression[148]. For example, PPARγ can be activated by dietary or intracellular fatty acids to antagonize NF-κB signaling pathway and inhibit the expressions of pro-inflammatory cytokines (**Figure 1.8**, page 40).

A few variants of PPARγ have been reported, including Pro12Ala (rs1801282), the first identified PPARγ polymorphism[149]. In this polymorphism, the proline (Pro) at codon 12 is substituted by an alanine (Ala), making PPARγ less active and less sensitive to its ligands[150]. A recent meta-analysis of 60 studies has found that Ala carriers are associated with a significant 14% lower risk of type 2 diabetes compared with Pro/Pro homozygotes[151]. The observed protective effect of Ala allele may be due to the less efficient activation of target genes which results in less accumulation of fat mass and improved insulin sensitivity[151]. Similarly, a 10-year follow up study among men with coronary artery disease reported inverse associations between carrying Ala allele and atherosclerosis, as well as 10-year vascular mortality and morbidity[152]. In contrast,

however, several studies also linked the presence of Ala allele to increased levels of body weight, BMI, waist circumference, or a tendency to gain weight over time among Ala carriers[153, 154]. Discrepancies of the study findings may be attributed to the influence of other genetic or environmental factors. Fatty acids (especially long-chain PUFA), as important PPARγ ligands, are of particular interest in this regard.

Evidence is spare and inconsistent, however, in terms of the impact of Pro12Ala polymorphism on fatty acid metabolism and how that is related to CVD risk. Lindi *et al.* conducted a randomized clinical trial (RCT) in subjects with impaired glucose tolerance (IGT)[155]. They found that in the intervention group with a reduced total fat and SFA diet, none of the Ala homozygotes developed type II diabetes and they lost more weight during follow-up than Pro allele carriers; whereas in the control group, Ala allele was linked to type II diabetes[155]. And interestingly, Goyenechea *et al.* [156] reported that under a 10-week dietary total fat intervention (i.e. 30% of energy as fat), PPARG Pro12Ala could strengthen the effect of IL-6 gene polymorphism on weight maintenance, although by itself it did not have any significant influence[156]. The inconsistent findings about the effect of Pro12Ala polymorphism may be due to the difference in study design, population, and type of fatty acids, especially each of the individual fatty acids. Thus far, there is no study addressing whether the fatty acid-inflammation relation differs by Pro12Ala polymorphism.

1.6 Mediation effect of inflammation and oxidative stress

Although it has been widely proposed that fatty acids (in diet or blood) may influence CVD risk via promoting or preventing inflammation[40-4240, 40]

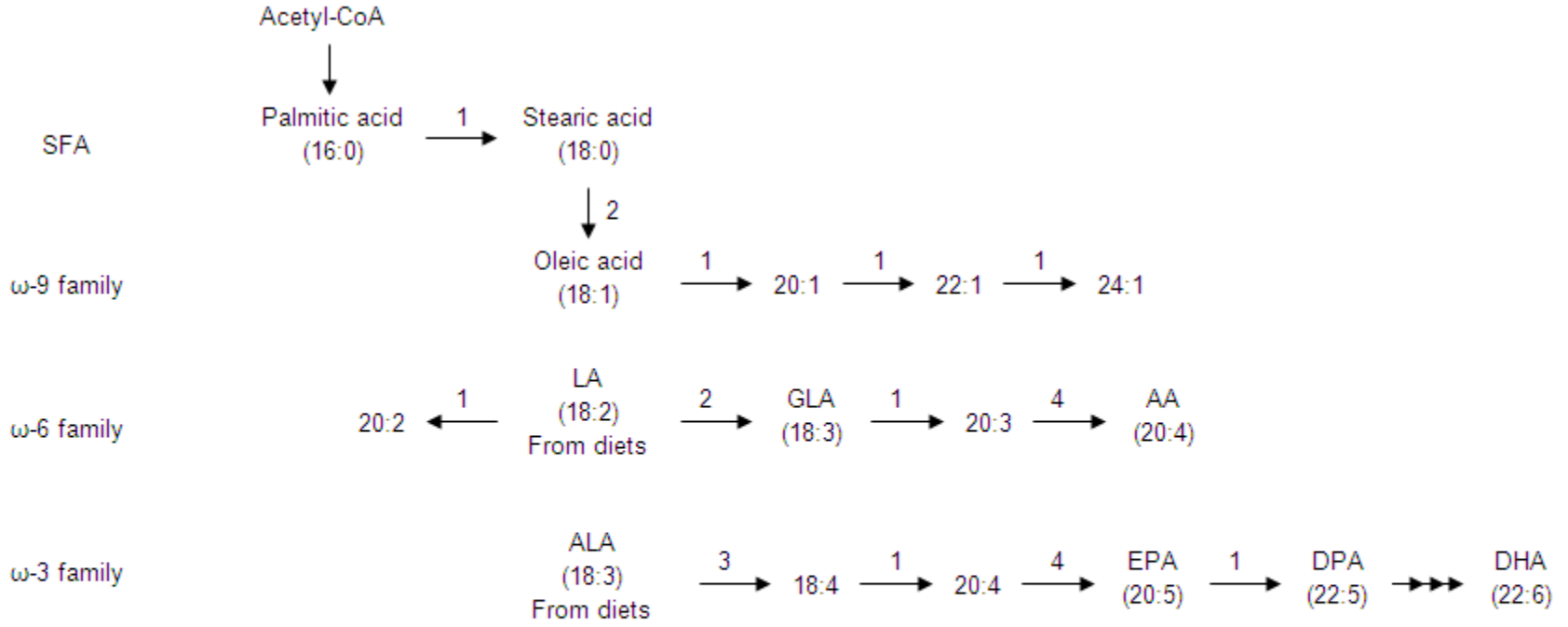
(Hall 2009; Hall 2009), there is no study, to date, examining the mediation effect of inflammation on the fatty acids-CVD association in a free-living population. Testing the mediation effect of inflammation may help explain the mechanism through which fatty acids affects CVD risk. Although causality may not be established from population-based prospective studies, exploring a mediation effect using observational data may provide information about potential mechanisms. A simple mediation model is described in **Figure 1.9** (page 41). In addition, by conducting this type of analysis, we can determine to what extent (complete or partially) does inflammation and oxidative stress mediate the fatty acids-CVD association. The potential mechanisms may be understood better, which may improve the design of future dietary intervention studies[157, 158].

Table 1.1 Commonly fatty acids found in diets or tissues

Type	Fatty acid	Systematic name	Common name
Saturated (SFA)	15:0	Pentadecanoic acid	N/A
	16:0	Hexadecanoic acid	Palmitic acid
	17:0	heptadecanoic acid	Margaric acid
	18:0	Octadecanoic acid	Stearic acid
Monounsaturated (MUFA)	16:1, ω7	Hexadecenoic acid	Palmitoleic acid
	18:1, ω9	Octadecenoic acid	Oleic acid
Polyunsaturated (PUFA)	18:2, ω6	Octadecadienoic acid	Linoleic acid
	18:3, ω3	N/A	α-linolenic acid (ALA)
	18:3, ω6	N/A	γ-linolenic acid (GLA)
	20:3, ω6	N/A	Dihomo-γ-linolenic acid (DGLA)
	20:4, ω6	Eicosatetraenoic acid	Arachidonic acid (AA)
	20:5, ω3	Eicosapentaenoic acid (EPA)	Timnodonic acid
	22:6, ω3	Docosahexaenoic acid (DHA)	N/A

Adopted partially from: http://www.nutritiondata.com/topics/fatty-acids.

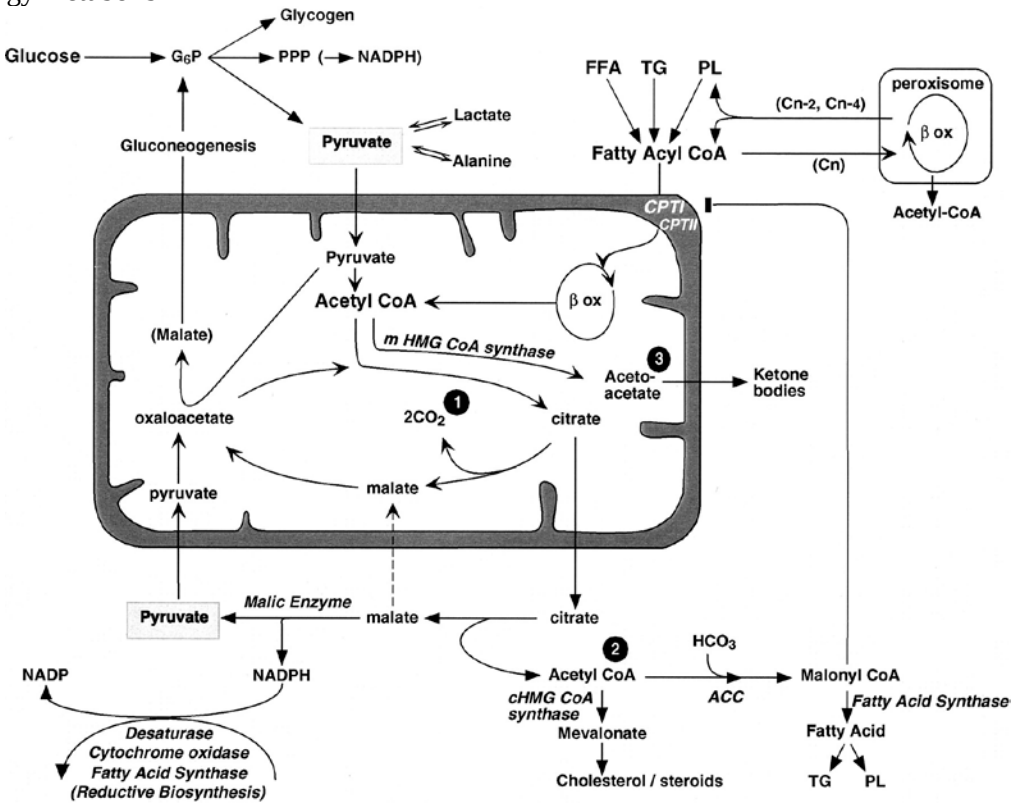
Figure 1.1 Biosynthesis of common fatty acids



1. elongase; 2. Δ^9 desaturase; 3. Δ^6 desaturase; 4. Δ^5 desaturase;
SFA: saturated fatty acid; LA: linoleic acid; ALA: α-linolenic acid ; GLA: γ-linolenic acid;
AA: arachidonic acid; EPA: eicosapentaenoic acid; DPA: docosapentaenoic acid; EPA: docosahexaenoic acid.

Adopted partially from Figure 23-9, Harper's Illustrated Biochemistry, 27th Edition

Figure 1.2 Fatty acids and energy metabolism



ACC: Acetyl CoA carboxylase; G6P: glucose 6 phosphate; PPP: pentose phosphate pathway; TG: triglycerides; PL: phospholipids; ß ox: ß oxidation; CPT: carnitine palmitoyl transferase.

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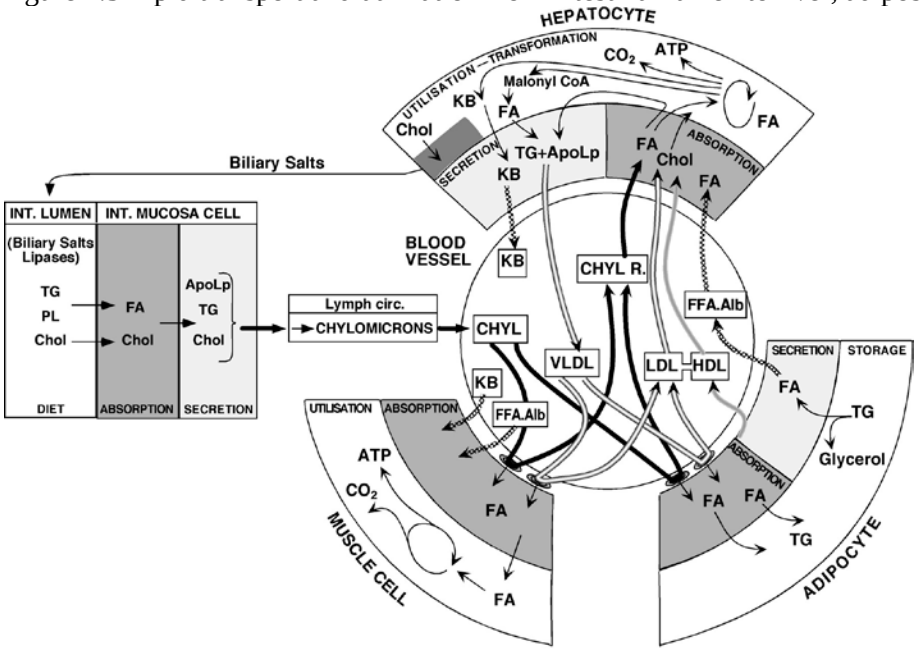
Figure 1.3 Lipid transport and utilization from intestinal lumen to liver, adipose, and muscular tissues



The absorption and processing of the lipids (TG, Chol, PL, FA) in the intestinal mucosa generate the CHYL, which circulate in the lymph and are delivered to the blood. FA uptake by muscle cells (energy substrate) and adipocytes (storage in form of TGs) after their release from TG by the extracellular lipoprotein lipase produces CHYL R., which are cleared from the blood by the hepatocytes. The FA reesterified in the hepatocytes are secreted in form of TGs associated with ApoLp in VLDL and LDL, which are depleted in FA by the action of lipoprotein lipase but enriched in Chol, as well as the HDL, which participate in the reverse transfer of cholesterol from peripheral cells to the liver, are taken up by hepatocytes. In energy-demanding situations, lipolysis in adipocytes triggers the release of FFA whose main part is transported in the blood by albumin (FFA.Alb), whereas the hepatocytes produce KB as fuel for peripheral tissues (muscle, brain, kidney).

TG: triglycerides; Chol: cholesterol; PL: phospholipids; FA: fatty acid; CHYL: chylomicrons; CHYL R.: chylomicron remnants;
ApoLp: apolipoproteins; VLDL: very low-density lipoproteins; LDL: low-density lipoproteins; HDL: high-density lipoproteins; KB: ketone bodies;

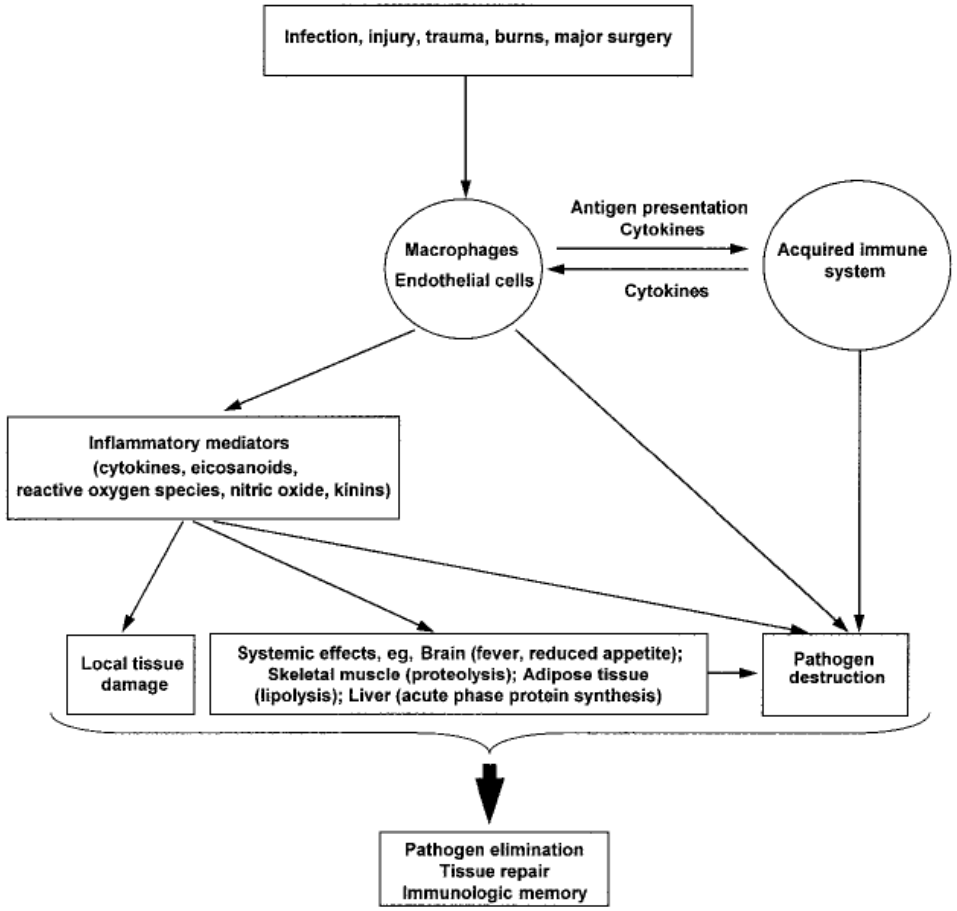
Endocrine Reviews 1999; 20 (5): 649

Table 1.2 Changes of hormone levels and FA metabolism in different tissues during the normal feeding and postprandial (starvation) phases

Tissues	Pancreas	Liver	Adipose tissue	Muscle
Feeding phase—taking nutrients from diets				
Changes of hormone levels and FA metabolism	Insulin level ↑ Glucogon level ↓	FA synthesis ↑ Cholesterol synthesis ↑ B-oxidation ↓ Ketogenesis ↓	FA synthesis ↑ (minor) TG synthesis ↑ Chylomicron uptake ↑ TG lipolysis ↓ B-oxidation ↓(minor)	FA uptake from chylomicron ↑ B-oxidation ↓
Postprandial (starvation) phase—FFAs and energy is needed				
Changes of hormone levels and FA metabolism	Glucogon level ↑ Insulin level ↓	B-oxidation ↑ Ketogenesis ↑ FA synthesis ↓	TG lipolysis ↑ B-oxidation ↑ (minor) FA uptake ↓	FA uptake from FFA in blood ↑ FA uptake from VLDL ↑ KB utilization ↑ TG lipolysis ↑ (minor) B-oxidation ↑ (minor)

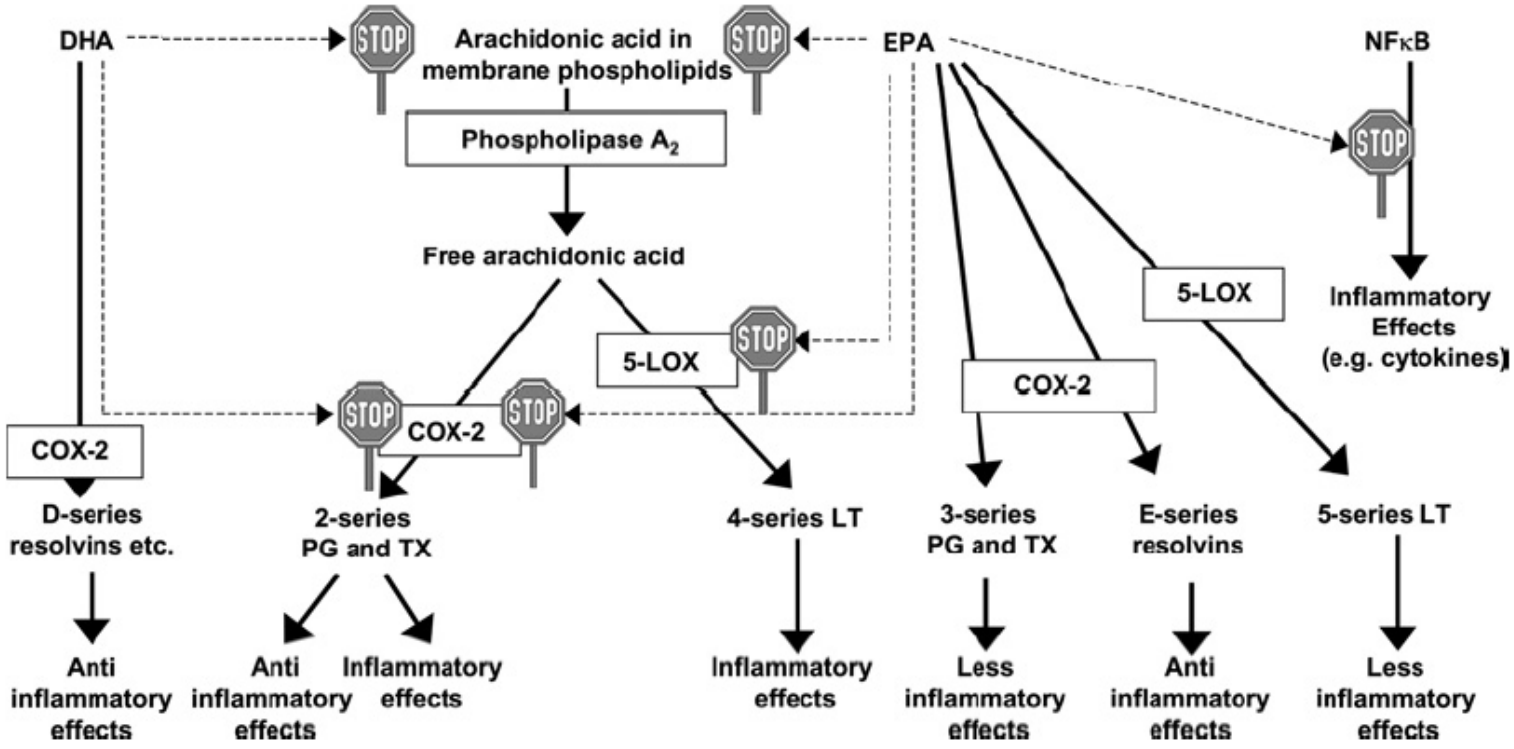
FA: fatty acids; FFA: free fatty acids; TG: triglycerides;↑: increase; ↓: decrease.

Figure 1.4 The role of inflammatory cells and mediators in regulating the whole-body metabolic and immunologic responses to infection and injury



Am J Clin Nutr. 2006; 83(6 Suppl): 1505S-1519S

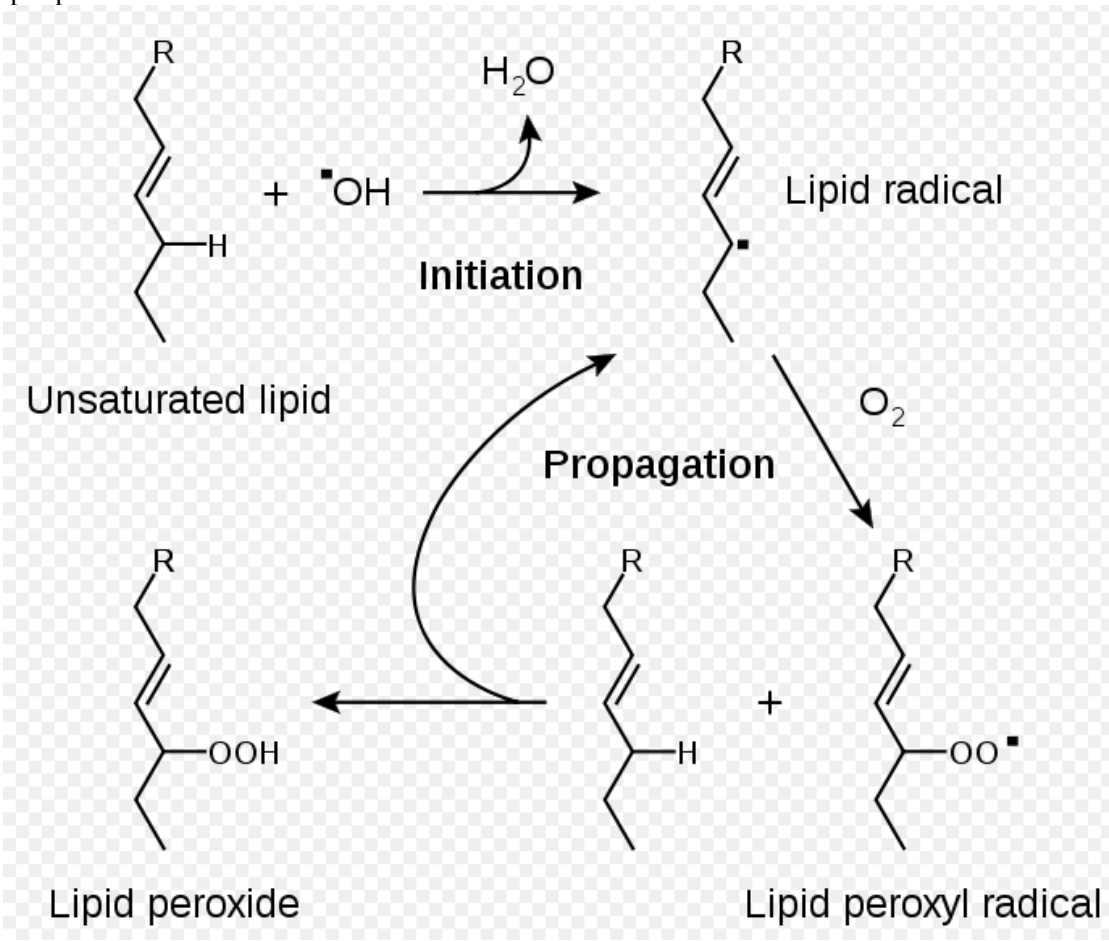
Figure 1.5 The current view of anti-inflammatory actions of long-chain ω3 PUFAs



PG: prostaglandin; TX: thromboxanes; LT: leukotrienes; COX: cyclo-oxygenase; LOX: lipoxygenase.

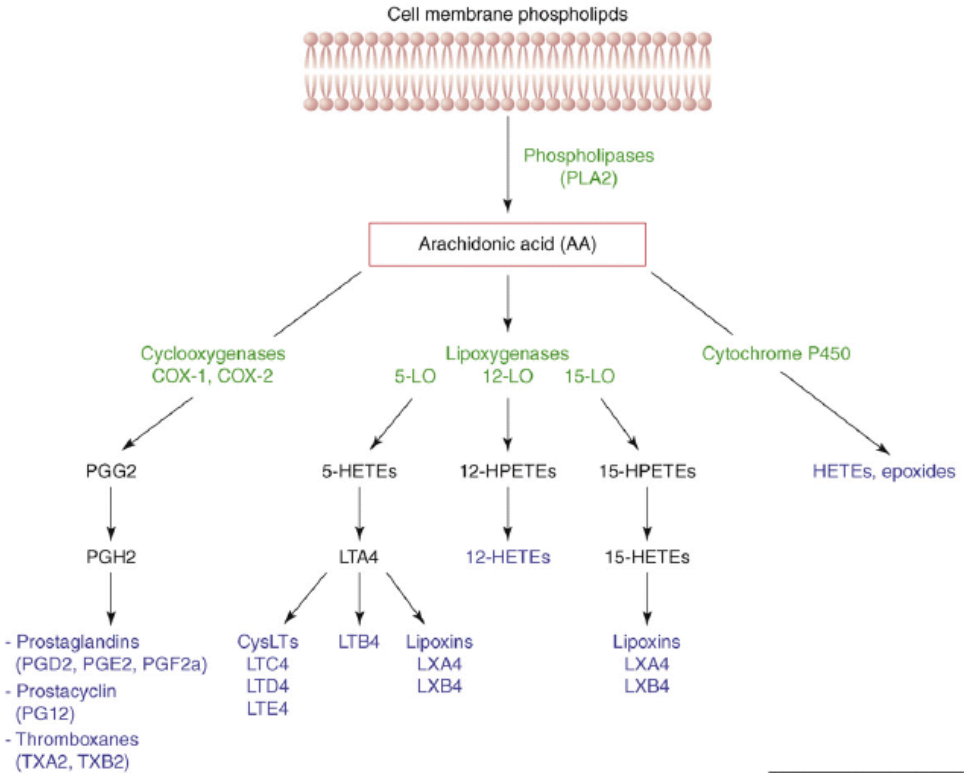
Biochem Soc Trans. 2005; 33(Pt2): 423-7

Figure 1.6 Mechanism of lipid peroxidation



From Wikipedia, the free encyclopedia “http://en.wikipedia.org/wiki/File:Lipid_peroxidation.svg”

Figure 1.7 Eicosanoid biosynthesis from AA



In response to a variety of non-specific activating stimuli, including cytokines, hormones and stress, AA is released from membrane phospholipids by phospholipases, especially cytosolic phospholipase A2 (cPLA2). Free AA can be converted to bioactive eicosanoids through the cyclooxygenase (COX), lipoxygenase (LOX) or P-450 epoxygenase pathways. LOX enzymes (5-LO, 12-LO, 15-LO) catalyse the formation of LTs, 12(S)hydroperoxyeicosatetraenoic acids and lipoxins (LXs), respectively. COX isozymes (constitutive COX-1 and inducible COX-2) catalyse the formation of PGH2, which is converted by cell-specific PG synthases to biologically active products, including PGE2, PGF2α, PGI2 and TXA2, known collectively as prostanoids. The P-450 exoxygenase pathway catalyses the formation of hydroxyeicosatetraenoic acids (HETEs) and epoxides.

Trends Mol Med. 2008; 14(10): 461-9

Table 1.3 Age-Related Changes in Cardiovascular Structure and Function

- *Structure*
  - Myocardial
    - Increased myocardial mass
    - Increased LV wall thickness
    - Increased deposition of collagen
  - Valvular
    - Increased thickness of aortic and mitral leaflets
    - Increased circumference of all four valves
    - Calcification of mitral annulus
  - Arterial
    - Increased intimal thickness
    - Increased collagen content
- *Function*
  - Heart rate
    - Decreased heart rate at rest
    - Decreased maximal heart rate during exercise
    - Decreased heart rate variability
    - Decreased sinus node intrinsic rate
  - LV systolic
    - Unchanged cardiac output
    - Increased stroke volume index
  - LV diastolic
    - Decreased LV compliance
    - Increased early diastolic LV filling
  - Myofibril
    - Unchanged peak contractile force
    - Increased duration of contraction
    - Decreased Ca^{++} uptake by sarcoplasmic reticulum
    - Decreased β-adrenergic-mediated contractile augmentation
  - Vascular
    - Decreased compliance
    - Increased pulsed-wave velocity

Mayo Clin Proc. 1996; 71(2): 184-96

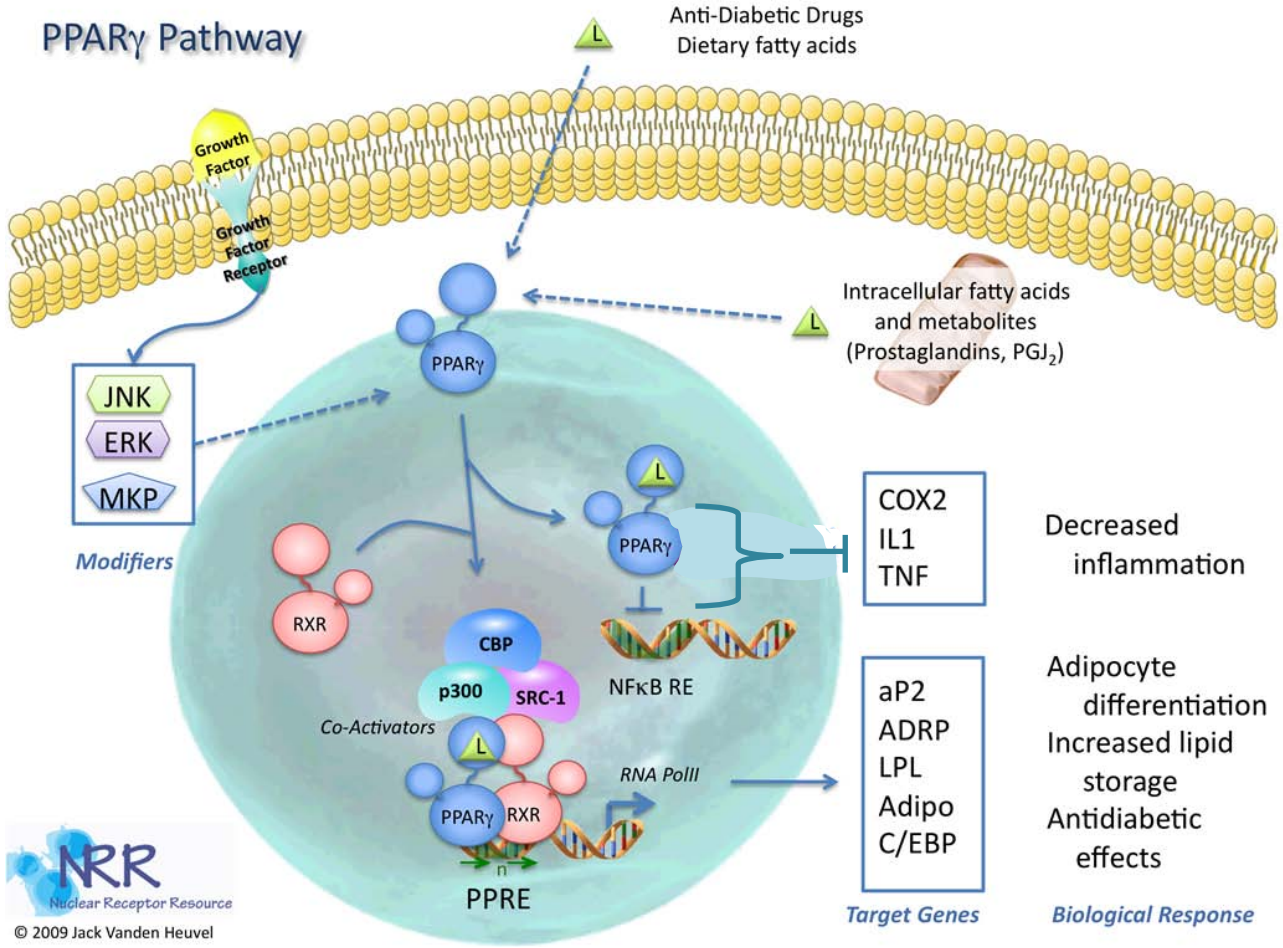
Table 1.4 Percentage of smokers by age group and sex in a large prospective cohort study with 14 786 Finnish participants

	25–49 Years			50–59 Years			60–64 Years			25–64 Years		
Cardiovascular Risk Factor	Men (n=4567), Mean	Women (n=4697), Mean	*P**	Men (n=1819), Mean	Women (n=1974), Mean	*P**	Men (n=704), Mean	Women (n=1025), Mean	*P**	Men (n=7090), Mean	Women (n=7696), Mean	*P**
Smoking, %	44.7	24.2	<0.001	35.5	11.4	<0.001	34.2	7.5	<0.001	41.0	19.0	<0.001
Cholesterol, mmol/L	5.94	5.51	<0.001	6.42	6.66	<0.001	6.42	7.00	<0.001	6.13	5.99	<0.001
HDL cholesterol, mmol/L	1.27	1.52	<0.001	1.24	1.52	<0.001	1.20	1.45	<0.001	1.26	1.51	<0.001
HDL/cholesterol ratio	0.22	0.28	<0.001	0.20	0.24	<0.001	0.19	0.21	<0.001	0.21	0.26	<0.001
Systolic blood pressure, mm Hg	140.8	132.3	<0.001	150.6	149.2	0.039	154.6	157.8	0.002	145.0	139.7	<0.001
BMI, kg/m^2	25.9	24.6	<0.001	27.4	27.8	0.002	27.3	29.0	<0.001	26.5	25.9	<0.001
Diabetes, %	1.7	1.4	0.241	6.7	5.0	0.023	8.1	9.5	0.312	3.7	3.3	0.174

*Difference between men and women, adjusted for age.

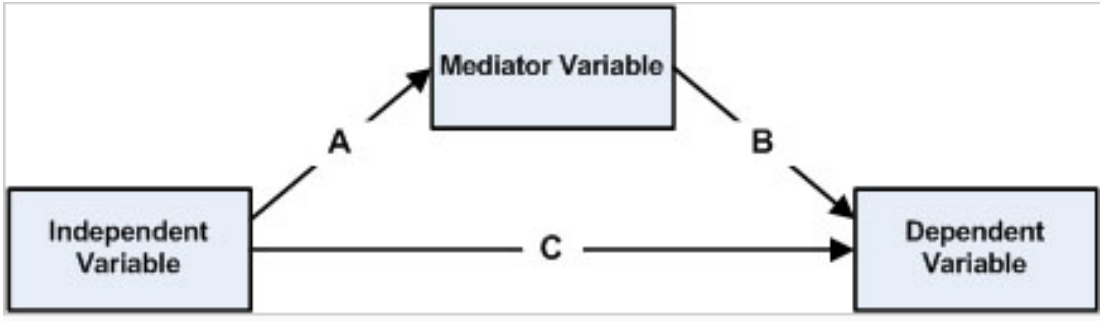
Circulation. 1999; 99(9): 1165-72

Figure 1.8 PPARγ pathway



Adopted from: http://nrresource.org/_Media/ppargamma_pathway-2.png

Figure 1.9 A simple statistical mediation model



From Wikipedia: http://en.wikipedia.org/wiki/File:Mediation.jpg

CHAPTER 2. STUDY DESIGN AND DATA COLLECTION

2.1 The Development of Insulin Resistance in Children Study

2.1.1 Study population and design

Data were collected in a prospective cohort study of obesity, insulin resistance and CVD risk factors in adolescents and was approved by the Human Subjects Committee of the University of Minnesota. Briefly, over 12 000 children in grades 5 to 8 enrolled in Minneapolis Public Schools were screened for blood pressure, height and weight in 1995. Participants were randomly selected from these children after stratification by sex, race (black and white), and systolic blood pressure[159]. Informed consent for participation was obtained from 401 children and their parents. Clinic visits were conducted at mean ages 13, 15, 19 and 23, with the number of the participants varying across visits.

2.1.2 Clinical measurements

A wall-mounted stadiometer and a balance scale were used to measure height (cm) and weight (kg), respectively. Body mass index (BMI) was calculated as weight in kg/height in m^2. Blood pressure was measured twice using a random-zero sphygmomanometer while participants were seated and the two values averaged in analyses. Tanner stage of children was assessed by a pediatrician according to pubic hair in boys and pubic hair and breast development in girls. The skinfold formula method of Slaughter[160] was used to calculate the % body fat, lean body mass (LBM) and fat-free mass. Dietary intake was assessed by a 127-item Willett food frequency questionnaire[161], from which the total energy intake for each participant was estimated. A modified Paffenbarger physical activity questionnaire was used to estimate energy expenditure[162,

[163]. Participants underwent euglycemic insulin clamp studies after overnight fasting with insulin sensitivity reported as M_{lbm} (mg of glucose utilization per kg of lean body mass per minute), as previously reported[159].

2.1.3 Laboratory measurement

Fasting blood specimens were collected immediately before the euglycemic insulin clamp studies for fasting insulin, glucose and lipids and stored at -70° C until analyses[164]. The serum fatty acid profile was analyzed by gas-liquid chromatography as described previously[165]. Individual CE and PL fatty acids were expressed as % total CE and PL fatty acids, respectively. Six biomarkers for inflammation and oxidative stress were analyzed, including adiponectin, CRP, 15-keto-dihydro-PGF2α, cytokines (IL-6 and TNF-α), and F2-isoprostanes. Adiponectin, IL-6 and TNF-α were measured using a noncommercial ELISA assay developed by the cytokine laboratory at the University of Minnesota and comparable to the commercial Quantikine kit[32]. An ultrasentitive colorimetric competitive ELISA was used to measure serum CRP level[166]. Both 15-ketodihydro-PGF2α and F2-isoprostane were analyzed from overnight urine samples by radioimmunoassay, adjusted for urine creatinine concentration[24, 27].

2.2 The Coronary Artery Risk Development in Young Adults (CARDIA) Study

2.2.1 Study population and design

The CARDIA is a multicenter, prospective cohort study for investigating the evolution of CVD risk factors and subclinical atherosclerosis. Participants at baseline (1985 to 1986) were a cohort of 5115 young black and white adults (aged 18 to 30 years) and from

Chicago, IL; Minneapolis, MN; Birmingham, AL; and Oakland, CA. The baseline recruitment was done by stratifying on age, race, sex and education levels, with the overall baseline response rates ranging from 51% to 82%[167]. As of year 2010, seven examinations have been conducted, which were at baseline (year 0), year 2 (response rate: 90%), 5 (86%), 7 (81%), 10 (79%), 15 (74%), and year 20 (72%)[143]. Informed consents were obtained from all participants at each exam and the study was approved by each site institutional review committee.

2.2.2 Dietary intakes assessment

Dietary intakes were collected at baseline, year 7 and 20, through an interviewer-administered diet history questionnaire specifically designed for the CARIDA study[168, 169]. Participants were asked to self-report their dietary intakes in the past 28 days, including the frequency, amount, and food preparation methods. The validity and reliability of this diet history questionnaire has been evaluated among 12 selected nutrients, which was described in details previously[170]. And then the nutrients consumed per day were calculated for every participant using the nutrient database (version 36) developed by the University of Minnesota Nutrition Coordinating Center.

2.2.3 Measurement of other baseline covariates

The demographic characteristics of participants (e.g. age, race, sex, smoking status, medication use, etc.) were assessed during interviews[171]. Self-reported alcohol consumption was recorded as ml/per week[172] and a validated CARDIA Physical Activity History Questionnaire was used to collect data of participants' physical activity[173]. During clinical visits, participants were wearing light clothing and no shoes when being

measured on height (m, to the nearest 0.5cm) and weight (kg, to the nearest 0.2kg). A balance beam scale and a vertical ruler were used for the height and weight measurements, respectively; and then the body mass index (BMI) was calculated as kg/m^2. Participants were seated and rested for 5min before their resting systolic and diastolic blood pressure were measured in their right arms. A random zero sphygmomanometer was used, and the average of the second and the third measurements was recorded.

For each participant, a 5ml overnight-fasting blood sample was collected between 7am and 10am, divided into two EDTA-containing Vacutainer Tubes. Serum and plasma samples were prepared separately, shipped (in dry ice) to each research locations and stored at -70°C until analysis (within maximum four months after collection)[174]. The plasma total cholesterol, HDL and triglycerides were assessed enzymatically[175] at the Northwest Lipid Research Clinic Laboratory at the University of Washington (Seattle, WA). In particular, the LDL-containing lipoproteins were precipitated with dextran sulfate/magnesium chloride before HDL level was determined[176] and then LDL level was estimated from the Friedewald equation for participants with ≤400mg/dL triglycerides[177]. Fasting glucose and insulin levels were quantified by the hexokinase method and standard radioimmunoassay, respectively[174]. The validity and reliability of duplicate measurements were evaluated[178, 179].

2.2.4 Plasma fatty acids measurements

Plasma PL fatty acids were analyzed in Year 20 at the Collaborative Studies Clinical Laboratory at Fairview-University Medical Center (Minneapolis, MN). The EDTA

plasma samples for analyses had been stored at -70°C and the analyses were performed using previously described methods[180, 181]. Briefly, lipids were extracted from plasma by chloroform/methanol extraction. The thin layer chromatography was used to separate lipid fractions, i.e. CE, PL, FFAs, etc. Individual fatty acids were assessed by gas chromatography flame ionization and expressed as percentages of total fatty acids.

2.2.5 Measurements of biomarkers of inflammation and oxidative stress

A few biomarkers of inflammation and oxidative stress were measured in the blood samples collected. At the Department of Pathology, University of Vermont, serum CRP level was determined by high-sensitivity ELISA assays at year 7, 15 and 20, using a BN-II analyzer[19]. The measurements of fibrinogen were conducted for three times (year 5, 7 and 20). At year 5, the assay was done by Clauss method[182, 183], while for the year 7 and 20 measurements, the BN-II nephelometer (N antiserum to Human Fibrinogen; Dade Behring Inc.) was used[19]. The level of IL-6 was examined only at year 20 using a high-sensitivity ELISA assay (R&D Systems, Minneapolis, Minnesota)[184]. The internal and external quality controls were also done to evaluate the potential technical errors of the assays, the details of which could be found in previous reports[179].

2.2.6 PPARγ polymorphism analysis

At year 10, 3798 participants were genotyped on the Pro12Ala polymorphism of PPARγ by TaqMan assay (Applied Biosystems, Foster City, CA) under a careful quality control[185]. Participants without genotype data, compared to those who had the data, were more likely to be African Americans, smokers, men, and people having lower education levels but higher total fat intakes[186].

2.3 The Atherosclerosis Risk in Communities (ARIC) Study

2.3.1 Study population and design

The ARIC study is a prospective cohort study conducted in four US communities and designed to study subclinical and clinical atherosclerosis and CVD risk factors in middle-aged adults. The four study centers are: Forsyth County, NC; the city of Jackson, MS; selected suburbs of Minneapolis; and Washington County, MD. There were 15,792 participants enrolled at baseline in 1987-1989 and examinations were performed every 3 years, with annual follow-up contacts via telephone, thereafter. The four examinations were in 1987-89, 1990-92, 1993-95 and 1996-98. All ARIC protocols were approved by local institutional review boards and all subjects gave informed consent. ARIC participant data were collected through clinic visits and interviews as described previously[187].

2.3.2 Dietary intake assessment

An interviewer-administered 66-item FFQ, slightly modified from the version of Willett et al.[188], was used to assess usual dietary intake at baseline (visit 1), while at visit 3, a replicate dietary measurement was also taken among the surviving cohort[188, 189]. To calculate the fatty acids composition, several factors were taken into account; including the frequency of food consumption, the portion size of each food and the type/brand of fat ordinarily used for cooking/baking/frying. The Harvard database[188] was used to obtain the nutrient values for each food; however, data were not available for some individual fatty acids[9].

2.3.3 Plasma fatty acids analysis

Plasma fatty acid components were only analyzed for the Minnesota center, with 4009 participants. Blood collection was under standardized conditions for the ARIC study[190]. Fasting blood was collected into 10ml vacuum tubes containing EDTA and sent by courier within 3h to the University of Minnesota Hospital and Clinic Laboratory. The blood was centrifuged at 800 g for 10min at 4°C. Plasma was then separated and dispensed into two 1.5ml aliquots and frozen at -70°C until analyzed for fatty acid content by a single technician.

A detail description of the methods used to analyze plasma fatty acids was published previously[9, 191]. After thawing, 0.5mL plasma was extracted with 0.5mL methanol followed by 1.0mL chloroform under a nitrogen atmosphere. The lipid extract was filtered to remove protein. The CE and PL fractions were separated by thin-layer chromatography with a silica gel plate (Silica Gel H; Analtech, Newark, DE) and 2-stage mobile phase development, which consisted of solvents of petroleum ether, diethyl ether, and glacial acetic acid in ratios of 80:20:1 (by vol) and 40:60:1 (by vol), respectively. The plate was dried between development solvents and the second mobile phase was allowed to migrate for only one-half of the plate length. After re-drying, one lane was sprayed with dichlorofluorescein to visualize the CE, PL, triglyceride, and free fatty acid bands under ultraviolet light. The CE and PL bands were scraped into separate test tubes, and the lipids were converted to methyl esters of fatty acids by boron trifluoride catalysis. The methyl esters were then separated and measured on a Hewlett-Packard 5890 gas chromatograph (Hewlett-Packard, Avondale, PA) equipped with a 50m FFAP WCOT glass capillary column (J&W Scientific, Folsom, CA) and a flame ionization detector.

The identity of each fatty acid peak was ascertained by comparison of the peak's retention time with the retention times of fatty acids in synthetic standard of known fatty acid composition, Each fatty acid (% of total fatty acid) was quantified by integrating the area under the peak and dividing the results by the total area for all fatty acids. To minimize transcription errors, the data from the gas chromatogram was electronically transferred to a VAX computer (Digital Equipment Corporation, Minneapolis) for data analysis. Test-retest reliability coefficients (individuals sampled 3 times, 2 weeks apart) for various plasma fatty acids ranged from 0.50 to 0.93 for CE and from 0.31 to 0.89 for PL [8].

2.3.4 Measurement of inflammatory/hemostatic biomarkers

Assessment of biomarkers was conducted at visit 1[18]. Fasting blood specimens were obtained from participants and collected in tubes containing EDTA (lipids), sodium citrate (hemostatic assays), or a separating gel (glucose and albumin). White blood cell count was determined in the whole blood sample using a Coulter Counter. Plasma levels of fibrinogen levels were determined using the thrombin time titration method, with reagents obtained from General Diagnostics Organon Tchnica Co. The albumin level was measured using DART albumin reagent from Coulter Diagnostics, a division of Coulter Electronics Inc., Hialeah, FL.

2.3.5 Measurement of other baseline covariates

Trained interviewers ascertained basic demographic data, medical history and information about personal habits, including education level, smoking, physical activity (represented as "sport index score" obtained from Baecke et al[192]), medication use, and

family history. Serum total cholesterol[193] and triglycerides[194] were measured by enzymatic methods, and high-density lipoprotein (HDL) cholesterol[176] was measured after dextran-magnesium precipitation. Low density lipoprotein cholesterol (LDL) was calculated using the Friedewald equation[177]. Fasting serum glucose was assessed by a hexokinase/glucose-6-phosphatase dehydrogenase method. Body mass index (BMI, kg/m2) and waist-to-hip ratio (WHR) were computed using measurements of weight, height, and circumferences of the waist and hips. Sitting blood pressure was measured 3 times on each participant with a random-zero sphygmomanometer after a 5-minute rest[195]; the average of the 2nd and 3rd readings was used in the analysis[196]. Hypertension was defined as a systolic blood pressure ≥140mm Hg or a diastolic blood pressure ≥90mm Hg or current use of antihypertensive medications. Cigarette-year of smoking was calculated by multiplying the average number of cigarettes smoked per day by the number of years of smoking.

Prevalent cardiovascular disease (CVD) was defined as angina or intermittent claudication by the Rose questionnaire[197]; a self-reported, physician-diagnosed history of a heart attack or stroke; a prevalent Q wave on ECG; or a self-reported history of cardiovascular surgery or angioplasty. Prevalent diabetes mellitus was defined as an elevated serum glucose of 140mg/dL (fasting), of 200mg/dL (nonfasting), and/or a history of, or treatment for, diabetes.

2.3.6 Incident CHD and IS assessment

ARIC followed the cohort and ascertained CHD and IS events using standardized methods described previously[198, 199]. Briefly, participants were contacted annually,

identifying hospitalizations and deaths during the prior year, and trained abstractors surveyed discharge lists from local hospitals and death certificates from state vital statistics offices for potential cardiovascular events[200, 201]. Incident cases of CHD were defined as[202] (1) a definite or probable myocardial infarction (MI); (2) a silent MI; or (3) a definite CHD death.

Incident IS was defined according to published criteria based on the occurrence and duration of neurological signs and symptoms, the results of neuroimaging and other diagnostic procedures, and treatments provided. Strokes secondary to trauma, neoplasm, hematological abnormality, infection, or vasculitis were not counted, and a focal deficit lasting<24 hours was not considered a stroke[203].

Incident cardiac events tracked through year 2007 are available for the current research.

CHAPTER 3. MANUSCRIPTS

3.1 Manuscript 1-- Obesity modifies the relations between serum markers of dairy fats and inflammation and oxidative stress among adolescents

Pentadecanoic acid (15:0) and heptadecanoic acid (17:0), the dairy-specific saturated fatty acids have been inversely, while inflammation and oxidative stress have been positively related to the risk of cardiovascular disease (CVD). Both fatty acid metabolism and inflammation and oxidative stress may be influenced by adiposity. In the current cross-sectional analyses among adolescents (mean age 15 years), we determined whether overweight status modified the associations between dairy fatty acids (pentadecanoic acid [15:0] and heptadecanoic acid [17:0]) represented in serum phospholipids (PL) and markers of inflammation and oxidative stress. Six biomarkers for inflammation and oxidative stress were analyzed, including circulating adiponectin, C-reactive protein (CRP), cytokines interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α), and urinary 15-keto-dihydro-PGF2α (15-keto), and 8-iso-PGF2α (F2-iso). Generalized linear regression analyses, adjusted for age, gender, race, Tanner score, total energy intake and physical activity, revealed that PL dairy fatty acids were inversely associated with CRP, F2-iso and 15-keto in overweight, but not in normal weight adolescents (all p-interaction<0.05). However, higher level of PL dairy fatty acids was associated with lower IL-6 among all adolescents. Further adjustment for dietary intake of calcium, vitamin D, protein, total flavonoids, and omega-3 fatty acids did not materially change

the findings. Dairy-specific saturated fats, i.e. 15:0 and 17:0 fatty acids, may contribute to the potential health benefits of dairy products, especially for overweight adolescents.

3.1.1 Introduction

The obesity epidemic has been increasing over the past 30 years among adults, adolescents, and children[204]. Childhood obesity may track into adulthood, and predict adult disease [205]. Inflammation and oxidative stress are common factors linking obesity and cardiovascular disease (CVD) risk [206]. Although low levels of inflammation and oxidative stress are normal in everyday tissue repair and acute injury in healthy people, chronic elevated levels of inflammatory and oxidative stress markers, such as C-reactive protein (CRP), interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α) [16], 15-keto-dihydro-PGF2α (15-keto) [24], and 8-iso-PGF2α (F2-iso) [27], have been observed with excess adiposity in both adults and children and with CVD in adults [206-208]. Further, levels of adiponectin, a protein hormone that is exclusively secreted from adipose tissue, have been inversely related to CVD risk [29, 209].

Another contributor to the development of CVD is dietary fat intake [40], and saturated fatty acids (SFA) have been widely shown to be positively related to levels of inflammation and other CVD risk factors, such as high blood pressure, elevated total and LDL cholesterol, insulin resistance and metabolic syndrome [40, 48, 51, 58, 210]. However, not all individual SFA have the same physiologic effects [210]. In this regard, 15:0 (pentadecanoic acid) and 17:0 (heptadecanoic acid) fatty acids, which come primarily from dairy products, are of particular interest [210]. Elevated levels of 15:0 and 17:0 fatty acids in blood, as objective biomarkers for dairy intake [83, 84], have been linked to lower

insulin-resistance syndrome, triglycerides and risk of developing myocardial infarction among adults [89, 211].

Evidence is limited in youth regarding the associations of 15:0 and 17:0 fatty acids with CVD risk, other than a study showing that the proportion of 15:0 fatty acid in serum cholesterol esters was inversely correlated with total cholesterol level in healthy adolescents [88]. It is worth noting that adiposity, in addition to dietary fat intake, may influence the endogenous fatty acid profile and metabolism. For example, obese adolescents had lower ω3 serum polyunsaturated fats (PUFA) and higher serum SFA, compared to their lean counterparts [212], and in an earlier report steffen et al. observed that adolescent overweight was associated with an adverse serum fatty acid profile and specific patterns of desaturase activity [213].

In the present study among adolescents mean age 15 years, we aimed to determine whether 15:0 and 17:0 fatty acids in serum phospholipids (PL) were related to inflammation and oxidative stress, and whether these associations differed by the weight status of adolescents. We hypothesized that PL 15:0 and 17:0 fatty acids would be inversely associated with IL-6, TNF-α, CRP, 15-keto and F2-iso, but positively associated with adiponectin, and these relations would be enhanced in the overweight adolescents.

3.1.2 Methods

Study population

Data for the present study were collected in a prospective cohort study of obesity, insulin resistance and CVD risk factors in adolescents that was approved by the Human

Subjects Committee of the University of Minnesota. Briefly, over 12,000 children in grades 5 to 8 enrolled in Minneapolis Public Schools were screened for blood pressure, height and weight in 1995. Participants for the prospective study were randomly selected after stratification by sex, race (black and white), and systolic blood pressure [159]. Informed consent for participation was obtained from 401 children and their parents. Clinic visits were conducted at mean ages 13, 15, 19 and 23 years, with the number of the participants varying across visits. The present study includes 305 adolescents seen at mean age 15 years (year 3 of the study) with complete data, including serum fatty acid measurements, biomarkers of inflammation and oxidative stress, anthropometric measurements and laboratory studies.

Measurements

Clinical measurements A wall-mounted stadiometer and a balance scale were used to measure height (cm) and weight (kg), respectively. Body mass index (BMI) was calculated as weight in kg/height in m2. Overweight is defined as gender-age specific BMI≥85 percentile according to the CDC growth chart [214]. Blood pressure was measured twice using a random-zero sphygmomanometer while participants were seated and the two values averaged in analyses. Tanner stage of children was assessed by a pediatrician according to pubic hair in boys and pubic hair and breast development in girls. The skinfold formula method of Slaughter [160] was used to calculate the % body fat, lean body mass (LBM) and fat-free mass. A modified Paffenbarger physical activity questionnaire was used to estimate energy expenditure [162].

Diet assessment Dietary intake was assessed by a 127-item Willett food frequency questionnaire (FFQ) [161], from which intakes of total energy and nutrients for each participant were estimated.

Laboratory measurement Fasting blood specimens were collected and stored at -70° C until analyses [159]. The serum PL fatty acid profile was analyzed by gas-liquid chromatography as described previously [165]. Individual PL fatty acids were expressed as % total PL fatty acids. Six biomarkers for inflammation and oxidative stress were analyzed, including circulating adiponectin, CRP, cytokines (IL-6 and TNF-α), and urinary F2-iso and 15-keto. Adiponectin, IL-6 and TNF-α were measured using a noncommercial ELISA assay developed by the cytokine laboratory at the University of Minnesota and comparable to the commercial Quantikine ELISA kit (R & D systems, Minneapolis, MN) [32]. An ultrasentitive colorimetric competitive ELISA was used to measure serum CRP level [166]. Both 15-keto and F2-iso were analyzed from overnight urine samples by radioimmunoassay, and divided by urine creatinine concentration [24, 27].

Statistical analysis

Descriptive data are presented as mean values stratified by normal weight and overweight. Skewed data were log-transformed prior to analysis; results were back-transformed and presented as geometric means. 15:0 and 17:0 fatty acids were analyzed both individually and together (the sum of 15:0 + 17:0 represented 'dairy fatty acids'). Generalized linear regression models were used to evaluate the relations between levels of biomarkers of inflammation/oxidative stress, serum dairy fatty acids and weight status. Effect modification of weight status was tested by including an interaction term between

weight status and fatty acids in the model. When effect modification was statistically significant, the relations between PL dairy fatty acids and biomarkers of inflammation and oxidative stress were stratified by weight status. Otherwise levels of biomarkers of inflammation and oxidative stress were examined across quintiles of fatty acids among all participants. Potential confounders were controlled in the analysis by adjusting for age, sex, race, Tanner score, total energy intake, and physical activity; and further for BMI when appropriate. Statistical models were also adjusted for dietary intake of calcium, potassium, phosphorus, vitamins A and D, protein, and omega-3 fatty acids, which have been shown to be potential bioactive components in dairy; as well as the dietary intakes of total flavonoids, representing intake of fruit and vegetables associated with the dairy intake [215]. Statistical significance was considered at $p<0.05$. All analyses were conducted using SAS (version 9.2, SAS Institute, Inc, Cary, NC).

3.1.3 Results

Descriptive characteristics.

Characteristics of the participants are listed in **Table 3.1.1**. The majority of study participants were white (80.3%) and male (57.1%). Normal weight adolescents were older than those who were overweight. Compared to their normal weight counterparts, the overweight adolescents had higher BMI (28.4 kg/m^2 vs. 20.6 kg/m^2), greater waist circumference (91.0 cm vs. 73.7 cm) and %body fat (45.7% vs. 26.6%) (all $p<0.001$). However, the two groups did not differ in physical activity or dietary intakes of total energy and selected nutrients.

The mean level of PL dairy fatty acids in normal weight adolescents was 0.70% of total PL fatty acids, which was significantly higher (p=0.01) than that of overweight adolescents (i.e. 0.67% of total PL fatty acids). Overweight adolescents had higher levels of TNF-α (3.9 pg/mL vs. 3.6 pg/mL, p=0.04), CRP (0.95 ng/mL vs. 0.74 ng/mL, p<0.001) and F2-iso (0.36 vs. 0.31 nmol/mol creatinine, p=0.01) than normal weight adolescents, while the level of adiponectin was significantly higher in normal weight adolescents (15.0 mg/L vs. 11.9 mg/L, p<0.001).

Effect modification.

As shown in **Figures 3.1.1 A, B, and C** after adjusting for age, gender, ethnicity, Tanner score, total energy intake and physical activity, weight status significantly modified the relations between PL dairy fatty acids and biomarkers of inflammation and oxidative stress. Among overweight, but not normal weight adolescents, higher levels of PL dairy fatty acids were associated with lower CRP, F2-iso and 15-keto (all p-trend≤0.01). Similar patterns were also observed when 15:0 and 17:0 fatty acids were examined individually (data not shown), although there was no association between PL 15:0 fatty acid and 15-keto in either the normal or overweight group ($p_{interaction}$ =0.39). No effect modification of weight status was observed for the relation between PL dairy fatty acids and either TNF-α or IL-6. Further controlling for the dietary intakes of calcium, potassium, phosphorus, vitamins A and D, proteins, total flavonoids, and omega-3 fatty acids did not materially change these results.

PL 17:0 fatty acid was associated with adiponectin in opposite directions in the two weight groups, after adjusting for age, gender, ethnicity, Tanner score, total energy intake

and physical activity ($p_{interaction}$=0.03, **Table 3.1.2**). Levels of PL 17:0 fatty acid were positively related to adiponectin in overweight adolescents (p-trend=0.08). In contrast, the inverse association was observed between PL 17:0 fatty acid and adiponectin in normal weight group (p-trend=0.06). Further controlling for the dietary intakes of calcium, potassium, phosphorus, vitamins A and D, proteins, total flavonoids, and ω3 fatty acids strengthened these relations (p-trend=0.01 and 0.04 for overweight and normal weight groups, respectively).

Other associations of fatty acids with inflammation and oxidative stress

In main effects analysis and adjusting for gender, age, ethnicity, Tanner score, total calorie intake and physical activity (**Figure 3.1.2**), adolescents with the highest quintile level of PL dairy fatty acid had the lowest IL-6 (p-trend<0.001) compared to those whose PL dairy fatty acid level was in the lowest quintile. Individual 15:0 and 17:0 fatty acids followed the same pattern. Notably, these inverse associations were not significantly influenced after further adjusting for BMI or dietary intakes of nutrients (data not shown). Null results were observed for TNF-α in all analyses (data not shown).

3.1.4 Discussion

In this cross-sectional study, dairy fatty acids (i.e. 15:0 and 17:0 fatty acids) in serum PL were found to be inversely related to biomarkers of CRP, F2-iso and 15-keto only among overweight, but not among normal adolescents. However, IL-6 was inversely related to PL dairy fatty acids independent of participants' weight status.

Few studies have directly linked dairy fatty acids to inflammation and oxidative stress among adolescents. Our findings are in agreement, however, with previous studies of

adults showing that higher levels of dairy fatty acids in blood or adipose tissues were associated with decreased risk of CVD, e.g. lower triglycerides, and small dense LDL particles, and reduced risk of stroke, insulin-resistance syndrome and myocardial infarction [89, 211, 216, 217]. In contrast, a positive association between plasma 15:0 fatty acid and risk of ischemic heart disease was found in a study of 32,826 adult women [87]. A study in adolescents found that 15:0 fatty acid measured in cholesterol esters was inversely related to total cholesterol [88].

PL fatty acids are involved in cell membrane activities such as cell signaling transduction, molecular transport, and maintenance of membrane fluidity [218]. It has been shown that PL fatty acid profiles represent short-term (weeks) fatty acid consumption [9] and may be a more objective measure of fatty acids that cannot be synthesized by humans [219]. 15:0 and 17:0 fit this category of fatty acids, reflecting dietary intake of dairy products [83, 84]. It is known that overweight individuals under-report dietary intake, especially dietary fat[220]. Thus, measures of PL fatty acids are likely to provide a more accurate assessment of dietary fat.

Dietary consumption of dairy products, despite being rich in SFA, does not appear to be associated with CVD risk [221-223]. Limited evidence has shown that higher dairy product intake substantially suppresses oxidative stress and inflammation among overweight and obese adults [85]. However, neither the underlying mechanisms nor the specific dairy product components related to these effects, in terms of 15:0 or 17:0 fatty acids, have been defined. In the present study the inverse associations of PL dairy fatty acids with CRP, F2-iso, 15-keto and IL-6 remained stable even after the adjustment for dietary

intake of nutrients (e.g. calcium, omega-3 fatty acids, etc. [215]) that may be related to the beneficial effects of dairy products. However, residual confounding cannot be ruled out, including other nutritional components and behavior factors associated with dairy intake.

In examining the role of adiposity in the relation between SFA metabolism and inflammation and oxidative stress [51], we found that PL dairy fatty acids were inversely related to CRP, F2-iso and 15-keto only in overweight adolescents, but not in their normal weight counterparts. Despite non-significant trends of linear associations which seemed to be due to a threshold effect, higher adiponectin was related to an elevated proportion of PL 17:0 fatty acids among overweight adolescents. Further adjusting for other dietary nutrient intake tended to enhance such positive association. These results supported our hypothesis that adiposity may play a critical role in the cross-talk between endogenous fatty acids and biomarkers of inflammation and oxidative stress. The lower CVD risk factors in normal weight adolescents, compared to their overweight counterparts, may be one explanation for the difference between the two weight status groups [224]. It is known that increased macrophage recruitment to the adipose tissues which is mediated by obesity results in low-grade inflammation [51]. Consistently, we found that normal weight adolescents had lower percent body fat and subsequently, lower levels of CRP and F2-iso, but higher adiponectin than overweight adolescents. In previous studies, similar effect modification of weight status was shown that higher plasma ω-3 PUFA was associated with lower risk of metabolic syndrome and inflammation only in overweight, but not normal weight adolescents [224]. Additionally, among 330 adults aged 18+ years with BMI of about 20-40 kg/m^2, Makhoul et al. showed

that EPA and DHA in red blood cell was inversely related to levels of triglycerides and CRP among overweight and obese people [225].

Our study, for the first time, reported the interaction between PL dairy fatty acids and adiposity in relation to inflammation and oxidative stress. Nonetheless, compared to other fatty acids, such as EPA, DHA and other SFA, the absorption, distribution and metabolism of odd-numbered SFA (i.e. dairy fatty acids in this study) in human body have been less investigated. A study in the mouse demonstrated that compared to even-numbered fatty acids, odd-numbered fatty acids were more likely to accumulate in epididymal fat rather than being beta-oxidized in liver [226]. Meanwhile, overweight and obese individuals are well known to have greater production of free fatty acids in their adipocytes, but lower beta-oxidation and a higher rate of fatty acid uptake in other tissues [227]. Therefore, one may speculate that the preferred accumulation of dairy fatty acids may augment their potential beneficial effects. However, there is no evidence so far showing whether and how dairy fatty acids are involved in anti-inflammatory/anti-oxidative cell signaling network from a molecular mechanism perspective.

Interestingly, among the six biomarkers of inflammation and oxidative stress examined, the effect modification of weight status observed between dairy fatty acids and CRP, F2-iso and 15-keto was absent for IL-6 and null results were found for TNF-α in all the analyses. Further, our findings were also slightly different for individual 15:0 and 17:0 fatty acids, such as in relation to 15-keto and adiponectin. This may be due to the complicated production network of inflammatory and oxidative stress signals in response

to their activators. Future studies are warranted in order to elucidate the cross-talk between dairy fatty acids, adiposity and inflammation/oxidative stress.

The current analysis was cross-sectional and therefore limited the ability to draw any conclusion about temporality or causality of the relations. The sample size was relatively small; nevertheless there was sufficient power to detect significant associations between serum PL dairy fatty acids and biomarkers of inflammation and oxidative stress. Although statistically significant effect modification and fatty acid-biomarker relations may be due to chance, the majority of results after controlling for multiple comparisons were consistent with our proposed priori hypotheses. Assessing serum PL dairy fatty acids, instead of self-report dietary dairy fat intake, provided an objective evaluation of nutrient-CVD risk factor associations. Although residual confounding may still exist, we accounted for factors that may influence adolescents' fatty acid metabolism, as well as the possible bioactive components that may contribute to the beneficial effects of dairy foods. The examination of the modification effect of body weight status, a particularly relevant factor, considered the strong association between overweight and markers of inflammation and oxidative stress.

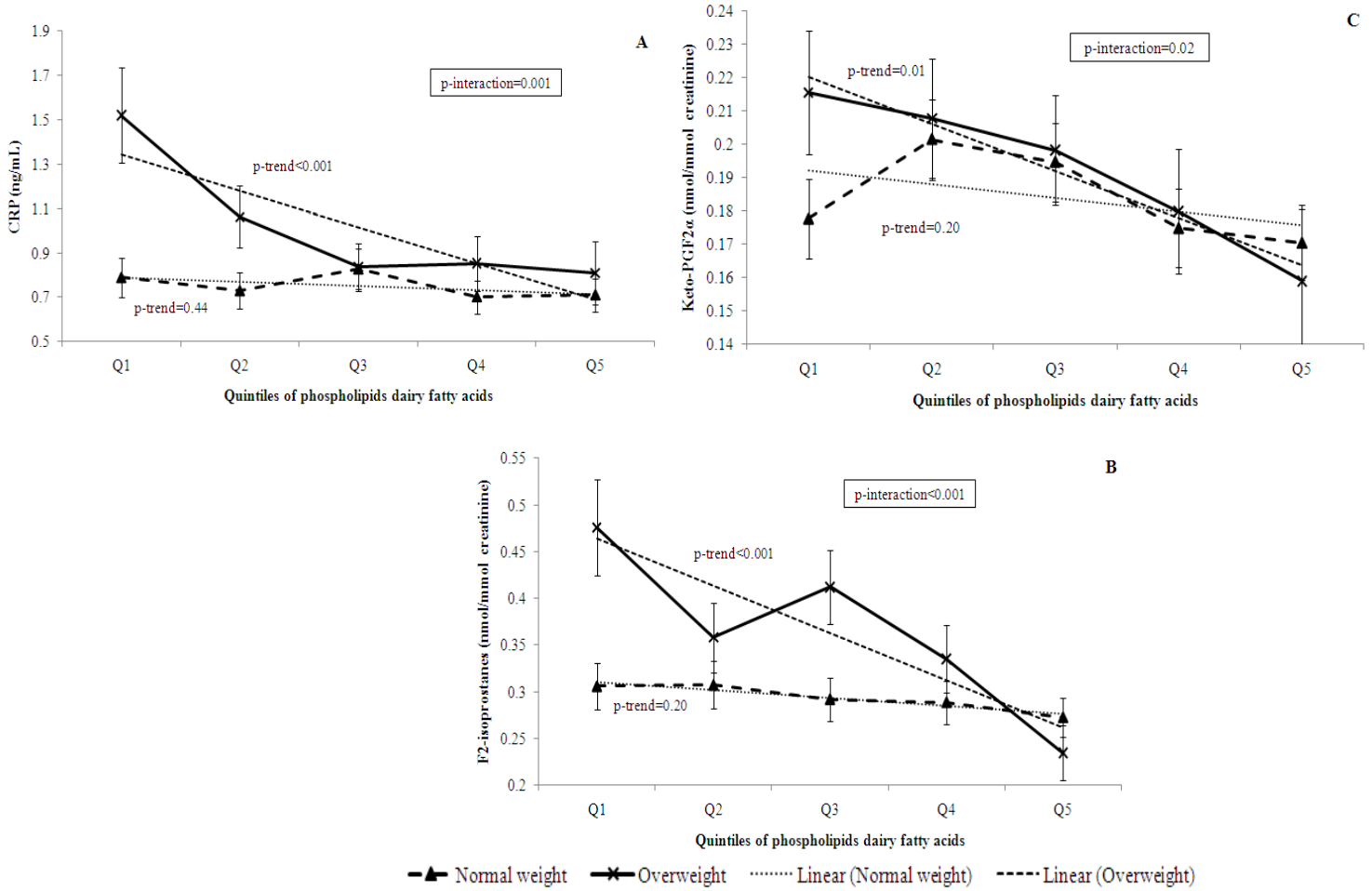
In summary, we observed that dairy fatty acids were beneficially associated with reduced CVD risk factors prior to adulthood in overweight, as opposed to normal weight, adolescents. These results support a goal of increasing dairy fatty acid intake early in life to set a dietary pattern consistent with preventing or delaying the development of CVD in later life [228].

Table 3.1.1 Unadjusted mean values of characteristics among normal and overweight adolescents† (n=305)

Characteristics	Normal weight (n=192)	Overweight (n=113)	p-value
Boys, n (%)	112 (58.3)	62 (54.9)	
Whites, n (%)	154 (80.2)	92 (80.5)	
	Mean±SD		
Age, years	15.2±1.2	14.7±1.2	<0.001
Tanner score	4.6±0.7	4.5±0.7	0.48
BMI (kg/m^2)	20.6±1.9	28.4±5.2	<0.001
Waist (cm)	73.7±7.1	91.0±13.3	<0.001
Lean body mass (kg)	44.3±9.5	42.8±9.2	0.18
% body fat	26.6±9.9	45.7±13.8	<0.001
Physical activity scores	5676±6370	5238±6251	0.56
Total energy intake (kcal)	2700±1377	2609±1371	0.57
Dietary intakes of nutrients‡			
Potassium (mg)	3223±119	3153±151	0.72
Phosphorus (mg)	1534±57	1559±75	0.79
Calcium (mg)	1106±49	1196±68	0.28
Vitamin A (IU)	9951±472	8843±548	0.13
Vitamin D (IU)	299±17	323±24	0.42
Omega-3 fatty acids (%kcal)	0.05±0.05	0.04±0.06	0.11
Total flavonoids (mg)	11±1	10±1	0.32
Phospholipids dairy fatty acids (%total phospholipids fatty acids)			
15:0 (pentadecanoic acid)	0.22±0.10	0.21±0.04	0.14
17:0 (heptadecanoic acid)	0.47±0.05	0.46±0.06	0.01
Dairy fatty acids (15:0+17:0)	0.70±0.12	0.67±0.08	0.01
Biomarkers of inflammation and oxidative stress			
Adiponectin (mg/L)	15.0±0.4	11.9±0.5	<0.001
IL-6 (pg/mL)‡	2.2±0.1	2.5±0.2	0.11
TNF-α (pg/mL)‡	3.9±0.2	4.6±0.3	0.04
CRP (ng/mL)‡	0.74±0.03	0.95±0.06	<0.001
F2-isoprostanes (nmol/mmol creatinine)‡	0.31±0.01	0.36±0.02	0.01
Keto-PGF2α (nmol/mmol creatinine)	0.19±0.01	0.20±0.01	0.12

† Overweight is defined as gender-age specific BMI≥85 percentile according to the CDC growth chart[214]
‡ Geometric means

Figure 3.1.1 The interactions between weight status and biomarkers of dairy fats in relation to inflammation and oxidative stress, adjusted for gender, age, ethnicity, Tanner score, total calorie intake and physical activity* (n=305)



*Geometric mean levels for CRP and F2-isoprostanes.

Table 3.1.2 Overweight modified the relation between adiponectin (mg/L) and quintiles of phospholipids 17:0 fatty acid among normal and overweight adolescents (n=305)*

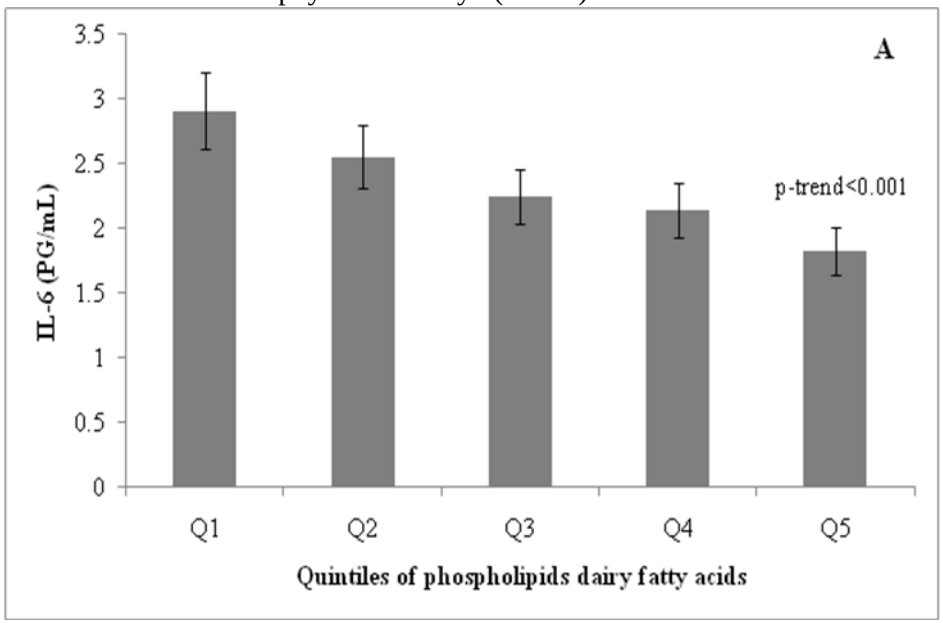
		Quintiles of phospholipids 17: fatty acid					
	Weight group	Q1	Q2	Q3	Q4	Q5	ptrend
Model 1†	Normal	16.1±1.1	17.0±1.0	14.3±1.0	14.5±1.0	14.5±0.9	0.06
	Overweight	10.5±1.0	11.6±1.0	12.9±1.0	12.8±1.2	12.5±1.3	0.08
Model 2‡	Normal	15.8±1.2	16.7±1.0	13.9±1.0	14.4±1.1	14.2±1.0	0.04
	Overweight	9.6±1.1	11.0±1.0	12.5±1.1	12.8±1.2	12.7±1.3	0.01

*$p_{interaction}$=0.03

† Adjusted for age, gender, race, Tanner score, total energy intake and physical activity

‡ Further adjusted for dietary intakes of calcium, potassium, phosphorus, vitamins A and D, proteins, total flavonoids, and ω3 fatty acids

Figure 3.1.2 The associations between biomarkers of dairy fats in serum phospholipids and IL-6, adjusted for gender, age, ethnicity, Tanner score, total calorie intake and physical activity* (n=305)



*Geometric mean level for IL-6

3.1.5 Supplemental results

In the additional analyses among this adolescent population, we extended Manuscript 1 by further examining whether weight status modifies the relations between other individual PL fatty acids and inflammatory biomarkers (i.e. IL-6, TNF-α, CRP, 15-keto and F2-iso). The detail methods have been described in Manuscript 1. Based on previous evidence, we hypothesized that PL 18:1ω9, 18:2ω6 and ω3 PUFA will be inversely associated, while 16:0, 18:0, 16:1ω7, 20:3ω6 and 20:4ω6 fatty acids will be positively associated with inflammatory biomarkers. These relations will be more pronounced in overweight group than normal weight group. Statistical significance was considered at $p<0.05$.

As shown in Table 3.1.3, Overweight adolescents had higher levels of PL 18:0 and 20:3ω6 fatty acids, but lower levels of PL 18:1 and 18:2ω6 fatty acids, compared to normal weight adolescents. In concordant with the findings for dairy fatty acids, weight status significantly modified the relations of PL 18:0 fatty acid with CRP, F2-iso and 15-keto (Pinteraction=0.03, 0.04 and 0.01, respectively), adjusting for age, race, Tanner score, total energy intake and physical activity. As shown in Figure 3.1.3 A, Serum PL 18:0 fatty acid was positively associated with CRP among overweight adolescents, but not in normal weight group (Ptrend=0.002 and 0.52 for overweight and normal weight group, respectively). Similar pattern was also found for the interactions between weight status and 18:0 fatty acid in relation to F2-iso and 15-keto (data not shown in the figure). In contrast, overweight adolescents who had higher factions of serum PL 20:4ω6 and 22:6ω3 fatty acids were observed to have lower IL-6 (Ptrend=0.02 and <0.001 for 20:4ω6

and 22:6ω3 fatty acids, respectively), whereas such inverse association was absent in normal weight adolescents (Figure 3.1.3 C and D, both Pinteraction<0.05). Notably, PL 20:3ω6 fatty acid tended to be positively related to IL-6 only in overweight group (Ptrend=0.03), but not in normal weight group (Ptrend=0.60), despite less statistical significance (Figure 3.1.3 B, Pinteraction=0.06). However, we were not able to detect significant modification effect of weight status on other fatty acid/inflammation relations (data not shown). Additionally, among all participants, by controlling for age, race, Tanner score, total energy intake, physical activity and BMI, PL 22:6ω3 fatty acid was found to be inversely correlated with F2-iso (Spearman correlation r=-0.15, p=0.02) and 15-keto (r=-0.17, p=0.008). PL component of 16:1ω7 fatty acid was positively correlated with F2-iso (r=0.15, p=0.02), while inverse correlation was found between PL 18:1ω9 fatty acid and CRP (r=-0.15, p=0.02). Notably, compared to the adjustment of only age, race, Tanner score, total energy intake and physical activity (data not shown), the additional adjustment of BMI enhanced the inverse correlations of 22:6ω3 and 18:1ω9 fatty acids with inflammation/oxidative stress, but slightly attenuated the positive correlations for 16:1ω7 fatty acid. Null results and weak correlations in the current analyses may be attributed to the relative small sample size of our study population.

This additional analysis further supported that adiposity may be critical in the cross-talk between endogenous fatty acids and biomarkers of inflammation and oxidative stress. Inflammatory biomarkers are generally elevated in excessive adipose tissues[206] and a few studies have shown altered lipid profiles and metabolism among overweight and obese individuals[229, 230]. The strengthened fatty acids/inflammation relations

observed among overweight adolescents than their normal counterparts may be partially explained by the greater variations of inflammation and fat metabolism. Notably, adiposity seemed to be more involved in the associations of SFAs with some specific inflammatory biomarkers, i.e. CRP, F2-iso and 15-keto, but not IL-6. This is surprising given the evidence showing that CRP and IL-6 are closely intertwined[231, 232], while significant results may be found by chance due to the paucity of the current sample. However, considering the complicated cell-signaling network, future studies are warranted to confirm these results and elucidate the underlying mechanism.

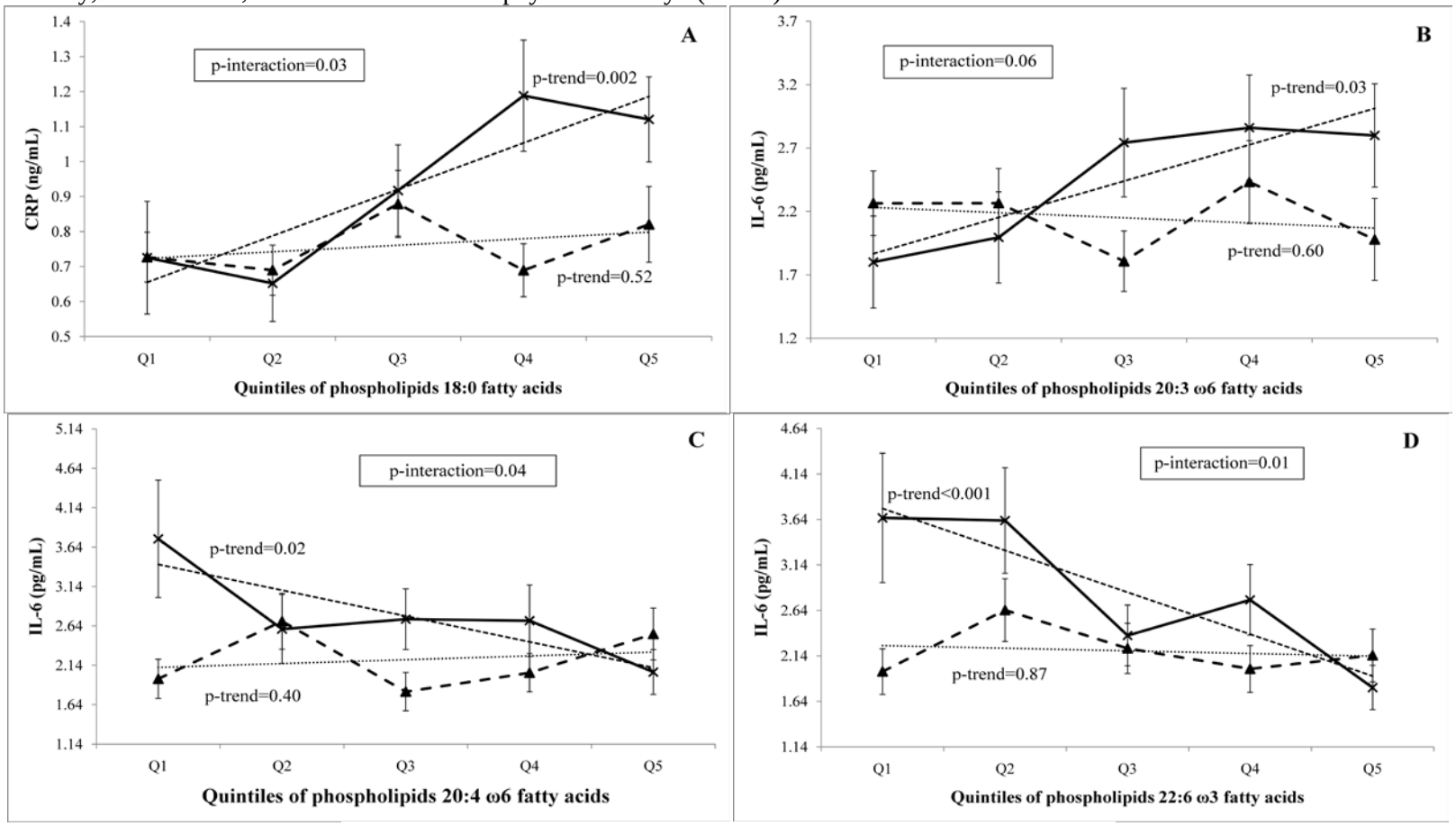
Results are further discussed in **CHAPTER 4. SUMMARY**.

Table 3.1.3 Unadjusted mean (±SD) values of individual fatty acids (%) in serum phospholipids among normal and overweight adolescents† (n=305)

Fatty acids (%)	Normal weight (n=192)	Overweight (n=113)	p-value
16:0	27.4±1.2	27.5±1.2	0.58
18:0	14.2±0.9	14.9±1.00	<0.001
16:1	0.84±0.20	0.86±0.20	0.45
18:1	14.1±1.6	13.5±1.4	<0.001
18:2 ω6	24.6±2.0	23.8±2.5	0.004
18:3 ω3	0.21±0.08	0.21±0.06	0.08
20:3 ω6	3.2±0.6	3.6±0.7	<0.001
20:4 ω6	10.6±1.5	10.9±1.6	0.10
20:5 ω3	0.44±0.29	0.45±0.13	0.13
22:6 ω3	2.3±0.6	2.4±0.5	0.19

† Fatty acids are presented as %total phospholipids fatty acids.

Figure 3.1.3 The interactions between weight status and serum PL fatty acids in relation to inflammation and oxidative stress, adjusted for gender, age, ethnicity, Tanner score, total calorie intake and physical activity* (n=305)



*Geometric mean levels for CRP and IL-6

3.2 Manuscript 2 – PPARγ Pro12Ala polymorphism modifies the associations of polyunsaturated fatty acids with inflammation: The Coronary Artery Risk Development in Young Adults (CARDIA) study

Intakes of polyunsaturated fatty acids (PUFA) have been related to inflammation, while less is known about the genetic variation modulating this association. This study aimed to explore whether the peroxisome proliferator-activated receptor γ (PPARγ) Pro12Ala polymorphism modified the relation between dietary PUFA intakes and the levels of inflammatory biomarkers (i.e. IL-6, CRP, fibrinogen). Gender-specific cross-sectional analysis was conducted among blacks and whites (n=2,647) completing the year-20 examination of the Coronary Artery Risk Development in Young Adults (CARDIA) Study. In both genders, the significant modification effect of Pro12Ala polymorphism was found for the relations between IL-6 (but not CRP or fibrinogen) and dietary intakes of arachidonic acid (AA, 20:4ω6) and long chain ω3 PUFA (LC-ω3, i.e. EPA 20:5ω3 and DHA 22:6ω3). The associations of IL-6 with both AA and LC-ω3 PUFA were positive in male Ala allele carriers, but were negative in female Ala carriers. Nothing was observed for female Pro/Pro homozygotes, whereas male Pro/Pro homozygotes who consumed higher LC-ω3 PUFA tended to have a lower IL-6 level. Independent of the effect of Pro12Ala polymorphism, dietary intake of α-linolenic acid (ALA, 18:3ω3) was inversely related to level of IL-6 in women, but not in men. In contrast, both of men and women who consumed higher percent of calories from AA were found to have higher levels of CRP and fibrinogen. These findings suggest that

dietary consumption of long chain PUFA may related to inflammation differently by PPARγ gene variants and in a gender-specific manner.

3.2.1 Introduction

Inflammation plays a central role in the development of cardiovascular disease (CVD), especially atherosclerosis, and its risk factors, such as insulin resistance, dyslipidimia, obesity, etc[233-235]. In contrast, lowering inflammation level may delay and prevent CVD risk[236]. Several biomarkers, including C-reactive protein (CRP), interleukin-6 (IL-6), fibrinogen, have been related to inflammation [20, 237] and the progression of CVD risk[237].

Dietary fatty acid intake, as reflected by blood fatty acid profile[9], is an important modifiable factor for promoting or preventing CVD risk[238]. Consumption of diets rich in polyunsaturated fatty acids (PUFA), especially very-long chain ω3 PUFA (e.g. EPA (20:5ω3) and DHA (22:6ω3))[58], contributes greatly to cardioprotection[239]. An advisory statement issued by the American Heart Association (AHA) also suggested that ω6 PUFA, when consumed in appropriate amounts, may be inversely related to CVD risk[115]. However, PUFA differ in metabolism and function and evidence is still inconclusive especially about the cardiovascular health effects of individual PUFA. For example, α-linolenic acid (ALA, 18:3ω3), an essential fatty acid for metabolizing into longer-chain ω3 PUFA in the human body, may not be as effective as EPA or DHA in preventing CVD risk [107]. In contrast to linoleic acid (LA, 18:2ω6), arachidonic acid (AA, 20:4ω6), as a substrate for various pro-inflammatory eicosanoids, has been commonly, but not always, linked to the promotion of pathological processes and inflammation [116, 122, 124]. Therefore, further study is warranted in evaluating the associations of different PUFA

with markers of inflammation. Notably, in addition to the diverse health effects of individual PUFAs, the genetic variability across study populations may be another explanation for the inconsistent findings on PUFA-CVD risk (including inflammation) relations.

Epidemiologic evidence has shown considerable individual variability in the development of chronic diseases in response to the same dietary components [141, 142]. Among the myriad of genes involved in lipid and glucose metabolism, peroxisome proliferator-activated receptor-γ (PPARγ) is also closely associated with inflammation and atherosclerosis[147]. As a member of the nuclear ligand-activated transcription factors [240], PPARγ could be activated by both of exogenous and endogenous natural fatty acids or synthetic drugs [241, 242]. Several PPARγ polymorphisms have been reported to alter disease progression via different gene functions from their wild-types [147]. Pro12Ala (rs1801282) is the first identified PPARγ polymorphism, in which the proline (Pro) at codon 12 is substituted by an alanine (Ala). This amino acid change makes PPARγ less active and less sensitive to its ligands [149, 150]. In a cross-sectional study, the associations of fish intake and ω3 fatty acids with glucose metabolism and fasting free fatty acids were modulated by (PPARγ) Pro12Ala polymorphism, partially via variation in the plasma proportion of DHA[243]. Similarly, the effect modification of Pro12Ala polymorphism was reported in a case-control study including 3,610 participants; the inverse association between dietary PUFA intake and risk of myocardial infarction found in Pro homozygotes was attenuated among Ala carriers [244]. However, the related evidence on such gene-diet interaction is still limited so far.

Therefore, in the current study among an African American and Caucasian young to middle-aged adult cohort enrolled in the Coronary Artery Risk Development in Young Adults (CARDIA) study, we examined the modification effect of PPARγ Pro12Ala polymorphism on the cross-sectional associations between dietary PUFA intake and inflammation. We hypothesized that the dietary consumptions of LA and ω3 PUFA (including ALA, EPA and DHA) would be inversely, whereas AA intake would be positively related to inflammatory biomarkers (i.e. IL-6, CRP and fibrinogen); and carrying Ala allele would attenuate these associations.

3.2.2 Methods

Study population

The CARDIA study is a multicenter, prospective cohort study for investigating the evolution of CVD risk factors and subclinical atherosclerosis. Participants at baseline (1985 to 1987) comprised a cohort of 5115 young black and white adults (aged 18 to 30 years) enrolled at field centers in Chicago, IL; Minneapolis, MN; Birmingham, AL; or Oakland, CA. The baseline response rates ranged from 51% to 82%[167]. Six follow-up examinations have been conducted at year 2 (average response rate: 90%), 5 (86%), 7 (81%), 10 (79%), 15 (74%), and year 20 (72%) [143]. Informed consents were obtained from all participants at each exam and the study was approved by each field center's institutional review board.

Assessment of dietary intakes

In the year 20 examination, dietary intake was collected through an interviewer-administered diet history questionnaire specifically developed for the CARDIA study[168].

Participants were asked to self-report dietary intake for the past 28 days, including the frequency, amount, and food preparation methods. The validity and reliability of this diet history questionnaire was evaluated among 12 selected nutrients, which was described in details previously[170]. Daily nutrient intake, including PUFA, was calculated using the nutrient database (version 36) developed by the University of Minnesota Nutrition Coordinating Center.

Measurement of other covariates

The demographic characteristics and lifestyle and medical information of participants (e.g. age, race, sex, smoking status, medication use, etc.) were assessed during interviews. Self-reported alcohol consumption was recorded as ml/per week[172] and a validated CARDIA Physical Activity History Questionnaire was administered[173]. During clinic visits, participants were wearing light clothing and no shoes when measured for height (m, to the nearest 0.5cm) using a vertical ruler and weight (kg, to the nearest 0.2kg) using a balance beam scale. Body mass index (BMI) was calculated as kg/m2. Participants were seated and rested for 5min before their resting systolic and diastolic blood pressure were measured with a random zero sphygmomanometer on the right arm. The average of the second and the third measurements was used in the analyses.

For each participant, a 5ml overnight-fasting blood sample was collected between 7am and 10am, divided into two EDTA-containing Vacutainer Tubes. Serum and plasma samples were prepared separately, shipped (in dry ice) to each research locations and stored at -70°C until analysis (within maximum four months after collection)[174]. The plasma total cholesterol, HDL and triglycerides were assessed enzymatically [175] at the

Northwest Lipid Research Clinic Laboratory at the University of Washington (Seattle, WA). In particular, the LDL-containing lipoproteins were precipitated with dextran sulfate/magnesium chloride before HDL level was determined [176] and then LDL level was estimated from the Friedewald equation for participants with ≤400mg/dL triglycerides[177]. Fasting glucose and insulin levels were quantified by the hexokinase method and standard radioimmunoassay, respectively [174]. The validity and reliability of duplicate measurements were evaluated [178].

Measurements of inflammatory biomarkers

Inflammatory biomarkers were measured in blood samples collected at year 20.. Serum CRP level was determined by high-sensitivity ELISA assays using a BN-II analyzer [19] at the Department of Pathology, University of Vermont. The BN-II nephelometer (N antiserum to Human Fibrinogen; Dade Behring Inc.) was used to measure fibrinogen [19]. The level of IL-6 was examined using a high-sensitivity ELISA assay (R&D Systems, Minneapolis, Minnesota)[245, 246]. The internal and external quality controls were conducted to evaluate the potential technical errors of the assays, the details of which have been described in previous reports[245, 246].

PPARγ polymorphism analysis

At year 10, 3,798 participants were genotyped on the Pro12Ala polymorphism of PPARγ by TaqMan assay (Applied Biosystems, Foster City, CA) under quality control[186]. The participants without genotype data, compared to those who had the data, were more likely to be African Americans, smokers, men, and people having lower education levels but higher total fat intakes[186].

Statistical analysis

All analyses were conducted using SAS (version 9.2, SAS Institute, Inc, Cary, NC). Participants were included in the current analyses who were genotyped on Pro12Ala polymorphism and also attended the year-20 examination (n=3,105). Among these, we further excluded those with missing measurements on dietary fatty acids (n=345), CRP (n=34), fibrinogen (n=78) and IL-6 (n=37). Participants who reported extreme energy intakes (<600 kcal or >6000 kcal per day for women or <800 kcal or >8000 kcal per day for men) were also excluded (n=399). After exclusion, a total of 2,647 (2647/3105= 85%) participants were included in the current analyses.

Individual dietary PUFA intake was expressed as percent of total energy intake (%kcal). A new variable "LC-ω3" was created by summing up the intakes of EPA and DHA. All PUFAs were treated as either continuous or categorical (tertiles) variables, while inflammatory biomarkers were treated as continuous variables in the analyses. Variables with skewed distributions were log-transformed before entering the analyses. Participants were grouped by their Pro12Ala polymorphism genotypes, that is, Ala carriers (Pro/Ala+Ala/Ala) and Pro homozygotes (Pro/Pro).

Since the effect of Pro12Ala polymorphism and fatty acid metabolism may both differ by gender[247-251], all analyses were performed separately by gender.

Generalized linear regression models were used to evaluate the interaction between Pro12Ala polymorphism and individual dietary PUFA intake in relation to inflammatory biomarkers. Model 1 controlled for potential confounders, including continuous variables: age, total energy intake, physical activity, alcohol intake, and categorical

variables: survey center (four centers), race (white vs. black), smoking status (ever vs. never), education level (less vs. more than high school), vitamin supplements use (yes vs. no) and cholesterol-lowering medication use (yes vs. no). Effect modification of Pro12Ala polymorphism was tested in Model 1 by including an interaction term (genotype × fatty acids (tertiles)) in the model. If effect modification was statistically significant, generalized linear regressions evaluated the relations between fatty acids and inflammatory biomarkers stratified by groups of Pro12Ala genotype. Otherwise, levels of inflammatory biomarkers were examined across tertiles of fatty acids among all participants. Linear trends in levels of inflammatory biomarkers across the fatty acid tertiles were also tested and generated by using the median fatty acid level in each tertile. In order to explore related biological mechanisms, a Model 2 was created by further controlling for BMI, systolic blood pressure, levels of HDL, LDL and triglycerides, and diabetes status.

All p-values were two-sided. Based on our priori hypothesis, interactions were considered significant at $p \leq 0.05$, whereas $p < 0.01$ was set for all other estimates, while $p < 0.05$ was also considered as borderline significant.

3.2.3 Results

Participants' characteristics

Participant characteristics at year 20 are shown in **Table 3.2.1** for both men and women by Pro12Ala polymorphism genotype. In both genders, Ala allele carriers were more likely to be older, white, physical active, highly educated, had lower blood pressure

and levels of inflammatory biomarkers, consumed less total calories and ω6 PUFA (i.e. 18:2ω6 and 20:4ω6), but more alcohol than non-carriers.

Modification effect of Pro12Ala polymorphism

In both genders, significant interactions were found between Pro12Ala polymorphism genotype and LC-ω3 intake in relation to IL-6 level, adjusting for age, survey center, race, education level, smoking status, total energy intake, physical activity, alcohol intake, vitamin supplements and cholesterol-lowering medicine use. As shown in **Table 3.2.2**, among men, the IL-6 level was lower in Pro homozygotes, but higher in Ala carriers along with the difference of dietary LC- ω3 consumption. In contrast, among female Ala carriers, those who consumed the lowest percent of calories from LC-ω3 were found to have the highest level of IL-6, whereas no such association between the dietary LC-ω3 intake and IL-6 was observed among female Pro homozygotes. Notably, there seemed to be a threshold effect, suggested by the non-statistically significant linear trend tests, for the inverse LC-ω3/IL-6 association among male Pro/Pro participants and female Ala carriers, as well as for the positive LC-ω3/IL-6 relation among male Ala carriers. Similar patterns were found for individual EPA and DHA relations with IL-6 level in each genotype group among both genders.

In addition to LC-ω3 PUFA, the association between dietary AA intake and IL-6 was also modified by Pro12Ala polymorphism in women, which followed the same pattern as that observed for LC-ω3. The above findings among women were further clarified and confirmed by the additional analyses, which combined the participants in dietary PUFA intake tertile 2 and 3 to compare with those in tertile 1.

Among men, IL-6 level was positively related to AA intake in both of male Ala carriers and non-carriers, which tended to be stronger among Ala carriers (P_{linear}=0.29 and 0.002 for Ala non-carriers and carriers across AA intake tertiles, respectively; $P_{interaction}$=0.03). However, although the association patterns maintained in the analyses combining tertile 2 and 3 to compare with tertile 1, the modification effect of Pro12Ala polymorphism on the relation between AA intake and IL-6 did not hold ($P_{interaction}$=0.30).

In contrast to IL-6, Pro12Ala polymorphism did not modify the associations of dietary AA and LC-ω3 intakes with levels of CRP or fibrinogen. Additionally, dietary consumption of LA and ALA did not interact with Pro12Ala polymorphism in relation to any of the inflammatory biomarkers.

Other PUFA-inflammation associations

Independent of the effect of Pro12Ala polymorphism, dietary ALA intake was inversely related to IL-6 level in women (P_{linear}=0.002), but not in men (P_{linear}=0.22) (**Figure 3.2.1**). Nevertheless, there was no association between ALA intake and levels of CRP or fibrinogen among both genders.

In contrast, as shown in **Figure 3.2.2**, male participants who consumed higher percent of calories from AA were found to have higher levels of CRP and fibrinogen (P_{linear}<0.001 and P_{linear} =0.002, respectively). Similar positive associations, with a threshold effect, were observed for women (P_{linear}=0.06 and 0.03 for CRP and fibrinogen, respectively).

Dietary intakes of LC-ω3 PUFA, including individual EPA and DHA, were not associated with levels of CRP or fibrinogen among men or women. There was no association between LA consumption and any of the inflammatory biomarkers examined.

All of the above findings were examined in Model 2 by further adjusting for BMI, systolic blood pressure, levels of HDL, LDL and triglycerides, and diabetes status, which, however, were not materially changed (data not shown).

3.2.4 Discussion

The associations between dietary PUFA intake and various CVD risk factors, including inflammation, have been widely investigated, whereas mixed results were shown across studies[48, 107, 114-116, 252, 253]. Genetic variations may be important contributors to these study discrepancies. Among the current middle-age adults, we showed that PPARγ Pro12Ala polymorphism significantly modified the association of dietary intakes of AA and LC-ω3 PUFA (including individual EPA and DHA) with level of IL-6, an important inflammatory biomarker.

PPARγ gene expresses primarily in adipocytes, but also distributes in several other tissues (e.g. liver, pancreas, etc.) or types of cells (e.g. macrophages, lymphocytes, etc.)[147]. By responding to its ligands and modulating the expressions of other genes, PPARγ has been identified as a potential anti-inflammatory factor, in addition to a regulator of energy homeostasis and lipid metabolism[147]. For example, PPARγ can interfere with several pro-inflammatory cell-signaling pathways by binding to key transaction genes of these pathways, such as NF-κB, AP-1, STAT-1, etc., and therefore suppress the expressions of various downstream pro-inflammatory genetic factors[147].

However, the variant of Ala allele at codon 12 reduces the binding ability of PPARγ to its downstream responsive DNA elements, resulting in decreased gene transcriptional ability[254]. It has been found that Ala allele is associated with lower lipoprotein lipase (LPL) activity, and thus reduced lipolysis[255]. In line with such *in vitro* evidence, Ala carriers have been found to have reduced plasma free fatty acids (FFA), improved insulin sensitivity and lower risk of type II diabetes[254, 256]. However, the population-based evidence regarding the association between carrying Ala allele and BMI is inconclusive, due to the potential higher fat accumulation resulting from lipolysis inhibition among Ala carriers[257]; whereas obesity has been linked to elevated inflammation and CVD risk[206].

Importantly, because of the variation of gene function between Pro and Ala alleles, Ala allele carries and non-carriers have been shown, when exposed to the same ligand, e.g. dietary PUFA, a group of well-documented PPARγ ligand, may experience different extent of CVD risk. In the current study, we observed that dietary intakes of AA and LC-ω3 PUFA were inversely related to IL-6 level among female Ala carriers, whereas such association was absent for female Pro homozygotes. In contrast, male Ala carriers who consumed greater AA and LC-ω3 PUFA had higher IL-6 level than those who consumed less AA and LC-ω3 PUFA. However, among male Pro homozygotes, similar but weaker positive association was found between dietary AA intake and IL-6, whereas higher intake of LC-ω3 PUFA was inversely linked to IL-6. Notably, the associations between PUFA intake and IL-6 tended to be stronger among the Ala carriers in the current study, which opposed to our *priori* hypothesis based on the fact that the Ala variant may be less sensitive to ligands than Pro allele[149, 150]. In a previous case-control study, consuming

each 5% higher calories from PUFA was associated with 34% significantly lower risk of MI among Pro/Pro participants, but only 7% lower (non-statistical significant) among Ala carriers[244]. However, several other previous studies supported our current findings, in terms of the interaction between Pro12Ala and PUFA intake in relation to CVD risk, as well as the strengthened relations among Ala carriers. For example, in a 3-month randomized clinical trial (RCT), Lindi et al. reported that with a fish oil supplementation intervention, Ala carriers had a greater decrease in serum triglycerides than Pro homozygotes when the total fat intake was ≤37%kcal or the SFA intake was ≤10%kcal[258]. Among 592 European adults enrolled in a cohort study, the ratio of dietary PUFA and SFA intake (P:S ratio) was shown to be inversely related to BMI and fasting insulin level among Ala carriers, while the opposite was seen among Pro homozygotes[259]. Nevertheless, Tai et al. and Vaccaro et al. both reported no significant interaction between P:S ratio and Pro12Ala polymorphism in relation to BMI or fasting insulin[260, 261]. In this regard, various types (i.e. ω3 vs. ω6) and individual PUFAs with different health effects may be an explanation for the controversies across studies.

Although increasing the intakes of EPA and DHA has been widely suggested for the purpose of CVD prevention, evidence is less clear and inconsistent regarding the ALA-CVD relation[107, 114]. Similarly, the recommendation of consuming at least 5%-10% of calories from ω6 PUFA was mostly based on evidence of LA[115]. AA may be positively associated with CVD risk due to its potential pro-inflammatory characteristics[116, 252, 253], whereas LA is the substrate for endogenous generation of AA. The diverse effects of individual PUFA on PPARγ have been documented *in vitro* studies. In human monocytic

cells, DHA activated PPARγ and downregulated the production of IL-1β, IL6 and TNFα (i.e. pro-inflammatory cytokines) more remarkably than ALA and LA[262]. Tishinsky et al. found that in cultured human adipocytes, the treatments of EPA and DHA had different impacts, partially or completely via PPARγ, on cellular adiponectin level, as well as the secretion of adiponectin[263]. However, the related population-based evidence is rare. Interestingly, among the current population, in contrast to AA and LC-ω3 PUFA, Pro12Ala polymorphism did not interact with dietary intakes of ALA and LA in relation to any examined inflammatory biomarkers. Future studies are warranted to confirmed such findings and further explore the variety of associations between Pro12Ala genotype and individual PUFA intake. Meanwhile, few studies so far examined how the PUFA-Pro12Ala interaction is related to inflammation. In addition to the present study, a cross-over RCT reported that a 4-week intervention of conjugated LA (CLA, a group of isomers of LA) affected, differently by Pro12Ala genotype, the expressions of several genes that may be related to inflammation, e.g. adiponectin, HPGD, etc[264]. As an important pathological condition for the development of CVD risk[235, 265], inflammation deserved more efforts in this regard.

It is noteworthy that among the three inflammatory biomarkers examined, only IL-6, but not CRP or fibrinogen, was related to PUFA intake via the modulation of Pro12Ala polymorphism; whereas, independent of PPARγ genotype, dietary AA intake was positively related to CRP and fibrinogen in both genders. Such discrepancies may be attributed to the complicated cell-signaling network, in which different genetic factors are involved in a variety of pathways. In another words, as supported by a few studies

(especially *in vitro*), the dietary PUFA may also be related to inflammation via the effect of toll-like receptor (TLR4) or directly modulating the endogenous fatty acid composition and metabolites, while bypassing the regulation of PPARγ[266]. And as reported by Herrmann et al. in their RCT with CLA intervention, not all gene expressions were PPARγ genotype-dependent[264]. On the other hand, a certain dosage of the PPARγ agonist (e.g. ω3 PUFA) may be necessary in order to attenuate the pro-inflammatory effects induced by CRP[267], although IL-6 is responsive for the hepatic CRP production[232] and CRP may promote inflammation by increasing IL-6 generation and reducing PPARγ expression[231]. However, the "trade-off" is that there seemed to be a threshold effect of dietary PUFA intake, as observed in the present and previous studies[268]. One possibility may be due to the threshold for incorporation of dietary PUFA into endogenous PUFA components[269]. This pool of evidence, therefore, suggested that consuming dietary PUFA to an appropriate amount may be important for achieving optimum cardiovascular health.

We found a significant gender-difference on the PUFA-Pro12Ala interaction in relation to inflammation; dietary intakes of AA and LC-ω3 PUFA were inversely related IL-6 level among female Ala carriers, but positively related to IL-6 among male Ala carriers. Such results were not surprising, however, since men and women have been shown can have substantial differences on both of fat metabolism and PPARγ function (including the function of Pro12Ala polymorphism)[247, 249-251, 270-272]. Consistent with our findings, Ylonen et al. reported that high fish intake and dietary EPA and DHA was associated with low fasting serum FFA only in male Pro homozygotes, but not in male Ala carriers; in contrast, only in female Ala carriers, but not female Pro/Pro, high fish

intake was inversely related to postprandial 2h glucose level[243]. In line with the gender-difference, the race-difference has also been widely proposed and examined. This is because that the population frequency of Ala allele varies among different ethnic groups, which is higher in Caucasians (12%) and Native Americans (10%), compared to that in African-Americans (3%) and Asians (<5%)[254]. In a previous CARDIA study, the Ala allele was related to lower average levels of fasting insulin, glucose and homeostasis model assessment only in African-American, but not in whites during 15-year follow-up[186]. Nevertheless, in the present study, we did not find significant race-difference on the PUFA-Pro12Ala interaction in relation to inflammation, which may be due to the relatively small number of black Ala carriers in this CARDIA population. In addition, there has been evidence showing that the effect of Pro12Ala polymorphism may also be influenced by the adiposity of study population[154, 273], whereas our study results were not materially changed by further adjustment for BMI and other clinical measurements.

Limitations of our current study included the possible measurement error of PUFA intake based on a 28-day dietary recall; the cross-sectional design, from which the causality of PUFA-inflammation association could not be established; potential under-power due to multiple-level stratification (i.e. by gender and genotype); and possible false positive results due to multiple statistical test conducted, although some *priori* hypothesis was set ahead. Meanwhile, it has been found that Pro12Ala polymorphism may be in linkage disequilibrium with a few other PPARγ variants, such as C1431T polymorphism[260], or interact with gene variants, such as IL-6 -174G>C polymorphism[156]

and β2-AR Gln27Glu polymorphism[274]. However, we were not able to rule out the confounding effects from other genetic variants in the current study.

In summary, among the current biracial middle-aged cohort, we reported gender-specific effect modification of PPARγ Pro12Ala polymorphism on the association between dietary PUFA intake and a specific inflammatory biomarker IL-6. Although there were some controversial results compared to previous evidence, our study suggested that dietary consumption of EPA and DHA may be particularly beneficial for female Ala allele carriers. However, because of the close intertwine between PUFA, PPARγ gene, and inflammation, future studies are warranted to confirm our findings, especially for dietary intakes of AA, ALA and LA, and the observations among men. The complicated underlying mechanisms should be further explored in order to better understand and explain all of these gene-diet-disease associations in free-living populations.

Table 3.2.1 Participants' characteristics at year 20 by gender and Pro12Ala polymorphism genotype: CARDIA (n=2,647)

Characteristics	Men		Women	
	Pro/Pro (n=951)	Ala carrier (n=198)	Pro/Pro (n=1,285)	Ala carrier (n=213)
Age (years)	45.2±3.5[a]	45.9±3.3	45.1±3.7	45.5±3.6
White (%)	54.0	87.9	46.1	84.5
Education level>high school (%)	74.1	82.8	75.6	86.9
Clinical factors				
Cholesterol-lowering medicine use (%)	12.4	14.4	7.0	5.7
Diabetes (%)	7.4	7.6	6.6	2.4
BMI (kg/m^2)	28.9±6.7	29.0±5.0	30.0±7.9	27.9±7.7
Systolic blood pressure (mmHg)	119.8±13.5	119.0±13.2	114.2±16.1	109.1±11.7
Plasma HDL-C (mmol/L)	47.6±14.6	47.6±14.4	58.9±16.1	61.0±15.9
Plasma LDL-C (mmol/L)	112.7±34.1	113.8±33.8	107.8±30.6	105.6±28.0
Plasma triglycerides (mmol/L)	125.8±87.1	130.8±95.8	93.8±62.0	90.5±57.0
Inflammatory biomarkers				
IL-6 (pg/mL)	2.4±3.0	2.3±2.6	2.8±3.0	2.4±3.4
hs-CRP (µg/mL)	1.9±4.3	1.4±1.8	3.5±5.2	2.7±5.0
Fibrinogen (mg/mL)	384±78	382±75	424±98	394±88
Lifestyle factors				
Ever-smoker (%)	35.9	35.0	36.7	48.6
Vitamin supplements use (%)	46.9	45.5	54.6	66.7
Physical activity (exercise units)	412±301	429±271	272±244	338±256
Total energy intake (kcal)	2736±1156	2651±980	2024±828	1998±768
Alcohol consumption (g)	13.2±21.4	15.3±18.7	8.1±16.5	9.7±16.2
PUFA intake (%kcal)				
18:2, ω6	7.05±3.01	6.84±3.0	7.36±3.35	6.49±2.96
18:3, ω3	0.777±0.425	0.787±0.442	0.880±0.532	0.762±0.408
20:4, ω6	0.061±0.032	0.056±0.031	0.058±0.030	0.054±0.026

20:5, ω3	0.023±0.033	0.024±0.040	0.026±0.035	0.024±0.031
22:6, ω3	0.044±0.048	0.047±0.063	0.048±0.054	0.048±0.051
LC-ω3	0.067±0.078	0.071±0.100	0.074±0.086	0.072±0.078

[a] Mean±SD for all such values

Table 3.2.2 Mean (±SE) levels of IL-6 across dietary fatty acid tertiles by Pro12Ala genotype: CARDIA [a]

		A priori analysis[b]					*A posteriori analysis*[c]				
		Fatty acid (%kcal) tertiles					Fatty acid (%kcal) categories				
		T1	T2	T3	*P*[d]	*P*[e]		T1	T2+T3	*P*[d]	*P*[e]
Men											
20:4, ω6		0.034[f]	0.055	0.083							
	P/P[d]	1.51±0.08	1.77±0.07	1.65±0,07	0.29	0.03	P/P	1.51±0.08	1.70±0.05	0.02	0.30
	A[d]	1.49±0.13	1.68±0.15	2.32±0.26	0.002		A	1.49±0.13	1.93±0.14	0.02	
20:5, ω3		0.003	0.014	0.038							
	P/P	1.73±0.05	1.70±0.07	1.51±0.06	0.02	0.03	P/P	1.73±0.05	1.60±0.05	0.16	0.001
	A	1.52±0.14	1.93±0.21	1.93±0.19	0.08		A	1.52±0.14	1.93±0.14	0.03	
22:6, ω3		0.012	0.028	0.073							
	P/P	1.77±0.07	1.58±0.06	1.57±0.06	0.13	0.004	P/P	1.77±0.07	1.58±0.05	0.04	0.01
	A	1.43±0.14	2.05±0.21	1.86±0.19	0.16		A	1.43±0.14	1.95±0.14	0.008	
LC-ω3		0.015	0.043	0.112							
	P/P	1.79±0.07	1.57±0.06	1.57±0.06	0.08	0.005	P/P	1.79±0.07	1.57±0.05	0.02	0.001
	A	1.48±0.13	1.95±0.20	1.93±0.19	0.07		A	1.48±0.13	1.95±0.14	0.02	
Women											
20:4, ω6		0.032	0.053	0.082							
	P/P	1.80±0.07	1.97±0.08	1.90±0.08	0.60	0.006	P/P	1.80±0.07	1.93±0.06	0.17	0.002
	A	2.14±0.19	1.51±0.14	1.62±0.16	0.10		A	2.14±0.19	1.55±0.10	0.006	
20:5, ω3		0.004	0.016	0.042							
	P/P	1.90±0.08	1.90±0.08	1.90±0.08	0.96	0.07	P/P	1.90±0.08	1.90±0.06	0.96	0.02
	A	2.10±0.19	1.60±0.14	1.55±0.16	0.21		A	2.10±0.19	1.57±0.11	0.01	
22:6, ω3		0.012	0.030	0.077							
	P/P	1.88±0.08	1.99±0.08	1.80±0.07	0.20	0.004	P/P	1.88±0.08	1.90±0.06	0.78	0.002
	A	2.25±0.23	1.48±0.15	1.58±0.16	0.09		A	2.25±0.23	1.54±0.11	0.001	
LC-ω3		0.017	0.046	0.121							

P/P	1.90±0.08	1.93±0.08	1.84±0.07	0.30	0.05	P/P	1.90±0.08	1.90±0.06	0.90	0.02
A	2.14±0.22	1.57±0.16	1.57±0.16	0.16		A	2.14±0.22	1.57±0.11	0.008	

[a] IL-6 levels are geometric means; model was adjusted for age, field center, race, education level, smoking status, total energy intake, physical activity, alcohol intake, vitamin supplements and cholesterol-lowering medicine use

[b] By fatty acids tertiles

[c] By fatty acids categories T1 vs. T2+T3

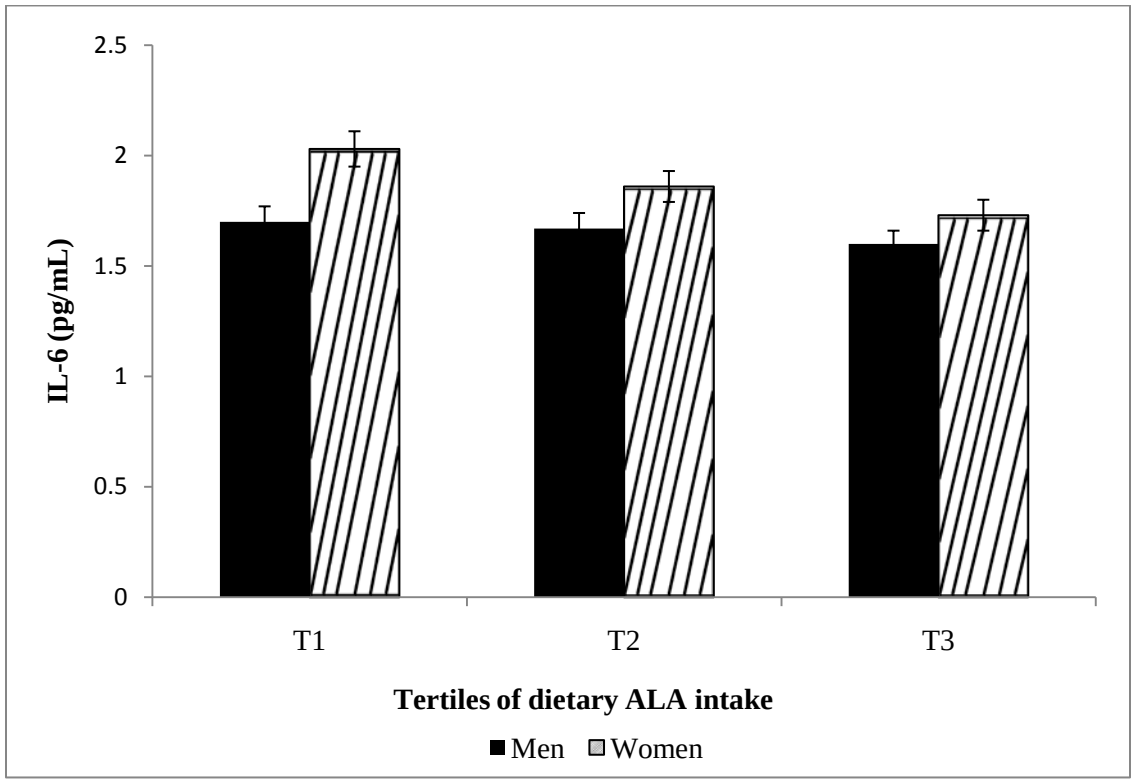
[c] For "By tertiles", P-values are for testing linear trend across fatty acid tertiles; for "T1 vs. T2+T3", P-values are for comparisons between fatty acid tertile 1 vs. tertile 2+tertile 3.

[e] P-values for testing interactions.

[f] Median fatty acid intake (%kcal) in each tertile (all such values)

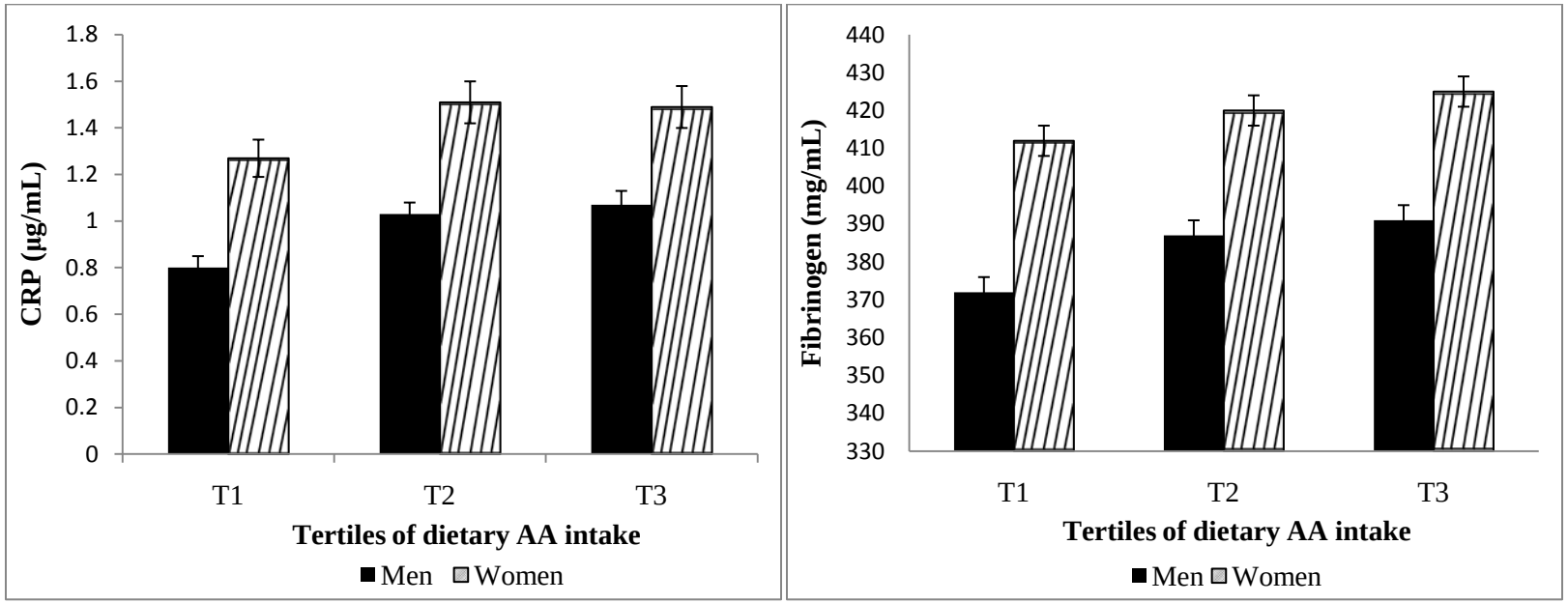
[g] P/P=Pro homozygotes; A=Ala carriers.

Figure 3.2.1 The associations between dietary ALA intake and IL-6 level among men and women: CARDIA [a] (n=2,647)



[a] Model adjusted for age, survey center, race, education level, smoking status, total energy intake, physical activity, alcohol intake, vitamin supplements and cholesterol-lowering medicine use;
IL-6 levels are geometric means.

Figure 3.2.2 Dietary AA intake is associated with levels of CRP and fibrinogen in both men and women: CARDIA (n=2,647)



[a] Adjusted for age, survey center, race, education level, smoking status, total energy intake, physical activity, alcohol intake, vitamin supplements and cholesterol-lowering medicine use;
CRP levels are geometric means.

3.2.5 Supplemental results

In addition to dietary PUFAs, we also examined the modification effect of Pro12Ala polymorphism for dietary SFAs (i.e. 16:0, 17:0 and 18:0 fatty acids) and MUFAs (16:1 and 18:1 fatty acids) in relation to inflammatory biomarkers, using the same methods as described herein. The hypothesis was dietary intakes of 16:0, 18:0 and 16:1 fatty acids would be positively, while 17:0 and 18:1 fatty acids will be inversely related to inflammation; and these relations would be more pronounced among Pro homozygotes than Ala carriers. Statistical significance was set at $p<0.05$.

However, the result was that Pro12Ala genotype did not significantly modify any of SFA/MUFA-inflammation relations (data not shown), after adjusting for age, field center, race, education level, smoking status, total energy intake, physical activity, alcohol intake, vitamin supplements and cholesterol-lowering medicine use. Independent of the effect of Pro12Ala polymorphism, among male participants, dietary intake of 16:1 fatty acid was positively, but very weakly, correlated to inflammation. The Spearman correlation coefficients r and the corresponding p-values were r=0.07, 0.12 and 0.11, and p=0.02, <0.001 and <0.001 for IL-6, CRP and fibrinogen, respectively. In women, however, positive and weak correlations were found between level of CRP, but not IL-6 or fibrinogen, with dietary intakes of 16:0, 18:0 and 16:1 fatty acids. Nevertheless, all of these weak correlations were attenuated after further controlling for BMI. In spite of some priori hypothesis for the fatty acids/inflammation relations, significant results may still be identified by chance due to the small magnitude.

Results are further discussed in **CHAPTER 4. SUMMARY**.

3.3 Manuscript 3 – Dietary fatty acid intake modifies the association of serum albumin with incident coronary heart disease and ischemic stroke: The Atherosclerosis Risk in Communities (ARIC) study

Dietary fatty acid intake has been closely related to the risk of cardiovascular disease (CVD), including inflammation and hemostasis. However, few studies have examined whether dietary fatty acid intake modulates the association of inflammation/hemostasis with the development of CVD. To explore the interactions between dietary fatty acid intake and inflammatory/hemostatic factors in relation to incident coronary heart disease (CHD) and ischemic stroke (IS) in the Atherosclerosis Risk in Communities (ARIC) Study. We hypothesized that higher intakes of saturated fatty acids and arachidonic acid (20:4ω6, AA) would interact synergistically, while increased consumption of ω3 polyunsaturated fatty acids and linoleic acid (18:2ω6, LA) would interact antagonistically, with hemostatic/inflammatory biomarkers in relation to incident CHD and IS. The ARIC cohort, including 15,792 middle-aged adults, has been followed since year 1987-89 (baseline visit). Inflammatory/hemostatic biomarkers, including plasma levels of von Willebrand factor (vWF), factor VIIIc, fibrinogen and white blood cell counts (WBC) and serum level of albumin, were measured and fatty acid intake was assessed by a food frequency questionnaire at baseline. After exclusions, 13,734 participants were included in the current analyses with 2,148 incident CHD events and 679 incident IS cases over 16 years follow-up. Cox proportional regression analysis showed that low versus high level of serum albumin was related to over 30% greater risk

of incident CHD among participants who consumed less LA or more AA. However, such associations were attenuated with increasing intake of LA or decreasing intake of AA ($P_{interaction}$=0.04 and 0.05, respectively). Similar patterns were found for the incident IS. There were no interactions between fatty acid intake and other hemostatic/inflammatory biomarkers in relation to CHD/IS incidence. Low serum albumin level, as a biomarker of inflammation and predictor of incident CVD events, may be susceptible to the modulation of dietary intake of LA and AA. Increasing consumption of LA and reducing AA intake to an appropriate extent may be recommended.

3.3.1 Introduction

Elevated hemostatic factors and chronic inflammation, which are closely intertwined, may promote the development of atherosclerosis. The underlying mechanism for atherosclerosis includes the recruitment of macrophages to the lesion site, expression and secretion of proinflammatory cytokines, and the formation of plaque on vascular walls[14, 16, 235, 275-277]. The thickening of arterial walls may eventually lead to incident coronary heart disease (CHD), as well as ischemic stroke (IS)[235, 278, 279]. Circulating biomarkers of hemostasis and inflammation, such as von Willebrand factor (vWF), factor VIIIc, fibrinogen, white blood cell counts (WBC) and serum albumin, etc., have been associated with cardiovascular events[16, 18, 37, 275, 277, 280-282].

Dietary fatty acids, as reported in a few studies, are actively involved in the progression of inflammation and cardiovascular diseases (CVD)[283-285]. Fatty acids may exert their health effects by modulating the pro-/anti-inflammatory markers[59, 286-288]. Generally, saturated fatty acids (SFA) are pro-inflammatory, resulting in increased risk of

CHD and IS[40, 238, 289]. However, ω3 polyunsaturated fatty acids (PUFA), especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are anti-inflammatory and cardioprotective[290-295]. Furthermore, individual fatty acids may have their specific effects. For example, stearic acid (18:0) may not be as atherogenic as palmitic acid (16:0)[81]. Although linoleic acid (18:2, LA) and arachidonic acid (20:4, AA) are both ω6 PUFA, AA has been commonly linked to the promotion of inflammation[116], which is in contrast to LA[296].

Compared to the relatively well-established relations of fatty acid with CHD, the evidence on the associations between fatty acids and IS is limited and inconsistent[72-74]. Among 79,839 women who were followed for 14 years, higher consumption of fish and ω3 PUFA was associated with a reduced risk of total stroke and thrombotic infarction, a subtype of IS[297]. Nevertheless, in a six-month randomized clinical trial conducted among 258 adults ages 45-70 years, Sanders et al. did not observe any difference in levels of IS-related hemostatic factors among study participants randomized to one of four diets with varying ω6/ω 3 fatty acid ratios[76]. In the Framingham Heart Study, higher intakes of total fat and saturated fatty acid (%kcal) were found to be associated with lower risk of IS in males[75]. Assuming that IS shares similar pathological mechanisms with CHD (e.g. inflammation and hemostasis), and since dietary intake is an important modifiable risk factor for CVD[298], a better understanding of the relations between dietary fatty acids, hemostatic and inflammatory biomarkers, and CHD/IS is warranted.

The current study, using data from a large prospective cohort study—the Atherosclerosis Risk in Communities (ARIC) study, aimed to investigate whether dietary

fatty acid intake modified the relation between risk of incident CHD/IS and levels of hemostatic/inflammatory biomarkers. Five biomarkers were examined, namely vWF, factor VIIIc, fibrinogen, WBC and serum albumin. We hypothesized that higher intakes of SFA and AA would interact synergistically, while increased consumption of ω3 PUFA and LA would interact antagonistically, with hemostatic/inflammatory biomarkers in relation to incident CHD and IS.

3.3.2 Methods

Study population

The Atherosclerosis Risk in Communities (ARIC) study is a prospective cohort study conducted to study subclinical and clinical atherosclerosis and CVD risk factors in middle-aged adults. The four study centers are Forsyth County, NC; the city of Jackson, MS; selected suburbs of Minneapolis; and Washington County, MD.

The baseline visit was conducted in 1987-1989, enrolling 15,792 participants. As described previously[187], all protocols were approved by local institutional reviewed boards and data were collected through clinical examination and interviews. Informed consent was sent to all participants. Three additional examinations were performed every 3 years after the baseline visit, with annual follow-up contacts thereafter.

Measurement of inflammatory and hemostatic biomarkers

Fasting blood specimen were drawn from ARIC participants' antecubital vein, from which the serum, plasma and whole blood samples were prepared and stored by standardized protocols in the ARIC hemostasis laboratory[199, 299]. Detailed information on the measurements of inflammatory and hemostatic biomarkers has been reported

elsewhere[299, 300]. WBC was determined in the whole blood sample using a Coulter Counter. Plasma levels of fibrinogen, vWF and factor VIIIc were determined by using the thrombin time titration method, ELISA kits and clotting assays, respectively[300]. The levels of vWF and factor VIIIc were then expressed as percent activity by relating the clotting time to a calibration curve constructed for each batch of samples. Serum albumin was measured with a Coulter DACOS instrument (Coulter Diagnostics, Hialeah, FL, USA) with a bromcresol green colourimetric assay[301].

Reliability of these measurements of inflammatory and hemostatic biomarkers was tested in subsamples of ARIC participants[302-304]. The reliability coefficients for fibrinogen, vWF, factor VIIIc, albumin and WBC were 0.72, 0.68, 0.86, 0.69 and greater than 0.96, respectively; whereas the intra- and interassay variability was all lower than 5%[18, 302-304].

Assessment of dietary intake

Dietary intake was assessed at both of baseline visit (visit 1) and year 6 (visit 3), using an interviewer-administered 66-item food frequency questionnaire (FFQ) slightly modified from the version developed and validated by Willett et al.[188]. Since biomarkers of inflammation and hemostasis were only measured at baseline, only baseline dietary intake data were used for the current analyses. Participants were asked how often on average in the past year they consumed a specific portion size of each food (e.g. 3-4oz of canned tuna fish). There were nine frequency categories: >6 (servings) per day, 4-6 per day, 2-3 per day, 1 per day, 5-6 per week, 2-4 per week, 1 per week, 1-3 per month, and almost never. The daily nutrient values were calculated by multiplying the nutrient

content of the specific portion size of each food (based on the Harvard nutrient database[188]) by the daily consumption frequency, and summing up all related food items. However, ω3 and ω6 linolenic acids (i.e. ALA and GLA) were not differentiated[9].

Alcohol intake was assessed by asking participants whether they currently drank alcoholic beverages, and, if not, whether they ever had drunk alcohol.

Measurement of other baseline covariates

Information of demographics, medical history, medication use and lifestyle habits, e.g. smoking, physical activity (Baecke 'sport index score'[192]), was obtained via trained interviewers administered questionnaires. Body mass index (BMI, kg/m^2) was calculated using measurements of weight and height. Sitting blood pressure was assessed three times on each participant after a 5-minute rest using a random-zero sphygmomanometer; the 2nd and 3rd readings were averaged for the analyses[195]. Hypertension was defined as a systolic blood pressure ≥140mm Hg or a diastolic blood pressure ≥90mm Hg or current use of antihypertensive medications. Fasting blood samples were used for lipid and glucose measurements. Plasma total cholesterol and triglycerides were measured by enzymatic methods[193], and high-density lipoprotein (HDL) cholesterol[176] was measured after dextran-magnesium precipitation. Low density lipoprotein cholesterol (LDL) was calculated using the Friedewald equation[193]. A hexokinase/glucose-6-phosphate dehydrogenase method was used to measure serum glucose and prevalent diabetes mellitus was identified with a serum glucose of ≥126mg/dL (fasting), of ≥200mg/dL (nonfasting), and/or a history of or treatment for diabetes. Prevalent CHD was defined at baseline as a (1) self-reported history of physician diagnosed heart attack; (2) previous

MI by ECG; (3) history of coronary bypass surgery; or (4) history of coronary angioplasty.

Assessment of incident CHD and IS

ARIC investigators followed the cohort and ascertained CHD and IS events using standardized methods described previously[198, 199]. Briefly, participants were contacted annually to identify hospitalizations and deaths during the prior year, and trained abstractors surveyed discharge lists from local hospitals and death certificates from state vital statistics offices for potential cardiovascular and cerebrovascular events. Incident cases of CHD were defined as (1) a definite or probable myocardial infarction (MI); (2) a silent MI; (3) a definite CHD death; or (4) a coronary revascularization[201]. Detailed information on the ascertainment and classification of stroke can be found elsewhere [305]. Diagnosis of stroke was based on the criteria adapted from the National Survey of Stroke and incident IS was indentified according to the evidence of acute infarction or no hemorrhage revealed by a brain photocopied neuroimaging (CT or MRI)[203, 305]. Incident cases of CHD and IS were tracked though year 2007.

Statistical analysis

All analyses were performed with SAS (version 9.2, SAS Institute Inc, Cary, NC). Participants were excluded from the analyses if they were non-white or non-black (n=48, due to limited representation); were blacks from the Minnesota and Maryland field centers (n=55, due to small numbers); had prevalent CHD (n=766) and stroke (n=286) at baseline; were missing measurements of vWF (n=273), factor VIIIc (n=281), WBC (n=246), fibrinogen (n=276) and albumin (n=150); were missing data of dietary fatty acid

and total energy intake (n=364); Participants who reported extreme energy intakes (<596 kcal or >3125 kcal per day for women or <697 kcal or >3763 kcal per day for men) were also excluded (n=513). These cutoffs represented approximately the lower and upper 1% of the energy-intake distribution. After all the exclusions, a total of 13,734 participants were included in the current study. The following analyses were conducted separately for CHD and IS.

Individual dietary fatty acid intake was represented as a percentage of total energy intake (%kcal) and grouped into three categrories (i.e. $\leq 25^{th}$ percentile, 25^{th} -75^{th} percentile, and $\geq 75^{th}$ percentile of each of the individual fatty acid distribution). A new variable "LC-ω3" was created by summing up the intakes of EPA and DHA. Inflammatory and hemostatic biomarkers were treated as either continuous or categorical (tertiles) variables. Variables with skewed distributions were log-transformed before entering the analyses and back-transformed to present as appropriate.

Cox proportional hazards models were used to estimate the hazards of incident CHD and IS in relation to inflammatory and hemostatic biomarkers, adjusting for age (continuous), sex (dichotomous), race-center group (categorical), total energy intake (continuous), drinking status (never-drinker vs. ever-drinker), smoking status (never-smoker vs. ever-smoker), physical activity (sport index score<2 vs. ≥ 2) and education level (more than high school vs. high school or less). Effect modification of dietary fatty acid intake was tested by including multiplicative interaction terms of fatty acid (categorical) x biomarker (continuous) in the model (Model 1). If significant effect modification of dietary fatty acid intake was observed, Cox proportional hazards

regression evaluated the associations between biomarkers (in tertiles) of inflammation and hemostasis and risk of CHD and IS stratified by categories of dietary fatty acid intake. Linear trends and the weighted average slopes (β coefficients) for risk of incident CHD and IS across the biomarker tertiles were tested and generated by using the median biomarker level in each tertile.

In the analyses stratified by dietary fatty acid intake, in addition to Model 1, other factors potentially involved in the biological pathways were also taken into account (Model 2), including continuous variables: BMI, systolic blood pressure, plasma HDL, LDL and triglycerides, and categorical variables: antihypertensive medication use (yes vs. no) and diabetes status (yes vs. no). For IS, Model 2 also adjusted for hormone replacement therapy use among women (categorical). Dietary consumption of carbohydrates and proteins, vitamin supplementation intake (yes vs. no), cigarette package years (continuous) and cholesterol-lowering medication use (yes vs. no) were also tested, but not included in the final analyses due to non-significant confounding effects. Further, cubic spline analyses were conducted in Model 1 for individual inflammatory and hemostatic biomarkers to evaluate their dose-response associations with incident CHD and IS by categories of dietary fatty acid intake. To minimize the influence of extreme values (i.e. $<1^{th}$ percentile or $>99^{th}$ percentile of the distribution) of inflammatory and hemostatic biomarkers, cubic spline analyses were performed only among sub-samples by excluding participants with extreme levels of specific biomarkers (i.e. for analyses of albumin, extreme levels of albumin were excluded). Knots were located at the 5^{th} , 25^{th} , 75^{th} and 95^{th} percentile of the biomarker distribution. The

reference value was set at the 90th percentile of the serum albumin distribution for the analyses of albumin, and set at the 10th percentile of the distribution for the analyses of other biomarkers.

In addition, an inflammatory biomarker risk score (IBRS) was created to combine information on levels of factor VIIIc, WBC, fibrinogen and serum level of albumin. For each participant, the values for each biomarker were expressed as a Z-score and the IBRS was calculated by summing up the four Z-scores[306]: $IBRS=(Z_{VIIIc}+Z_{WBC}+Z_{fibrinogen}-Z_{albumin})/4$. vWF was not included in the IBRS, because factor VIIIc is released from vWF by the action of thrombin when coagulation is stimulated and therefore factor VIIIc and vWF are highly correlated.

To evaluate a potential gender or race-center difference, we also tested the three-way interactions in models 1 by including multiplicative interaction terms (gender or race-center x dietary fatty acid (categorical) x biomarker (continuous)). However, none of these interactions was significant.

All p-values were two-sided. Proportional hazard assumptions were tested by including cross-product terms of the log function of follow-up time with biomarkers or fatty acids. To reduce problems related to conducting multiple comparisons, interactions were considered significant at $p\leq0.05$, while $p<0.01$ was set for all other estimates.

3.3.3 Results

Baseline characteristics of participants

Table 3.3.1 showed the unadjusted baseline characteristics of participants. The current study included 13,734 participants and the majority of them were white (74.5%) and

women (56.6%). Among those who developed incident CHD (n=2,148), 63.2% were men; and 51.3% of incident IS events occurred in men. Compared to participants who were free of incident CHD during follow-up, the incident CHD cases were more likely to be older, high-educated, ever-drinkers, ever-smokers, physical inactive, diabetic, consumed greater total calories and use anti-hypertensive medication. Other traditional CVD risk factors, including BMI, systolic blood pressure and plasma LDL and triglycerides, were also higher, while plasma HDL was lower among participants who developed incident CHD than those who did not. A similar pattern was observed between incident IS cases and non-cases.. Compared to their non-CHD/IS counterparts, the participants who developed incident CHD or IS consumed more calories from SFA and MUFA.Similar to the traditional CVD risk factors, levels of the hemostatic and inflammatory biomarkers, including vWF, VIIIc, WBC and fibrinogen, were elevated among the participants with incident CHD and IS. However, baseline serum albumin level was slightly lower in IS cases than IS non-cases, whereas no such difference was observed between two CHD groups.

Incidence of CHD and IS

During an average of 16.5 years follow-up, a total of 2,148 participants developed incident CHD. The overall crude CHD incidence rate was 9.5 per 1000 person-years. Meanwhile, a total of 679 participants developed incident IS over an average of 17.3 years follow-up. The overall crude incidence rate of IS was 2.9 per 1000 person-years.

Assessment of effect modification in relation to incident CHD and IS

Dietary fatty acid intake did not significantly modify the associations of vWF, factor VIIIc, WBC and fibrinogen with incident CHD. However, significant interactions were found between serum albumin level and dietary intake of LA and AA in relation to incident CHD ($P_{interaction}$=0.04 and 0.05), adjusting for age, gender, race-center, total energy intake, drinking status, smoking status, physical activity and education level (**Table 3.3.2**). Specifically, as shown by the β coefficients for trend, the inverse (despite lack of statistical significance) association of serum albumin with incident CHD was attenuated by greater intake of LA. In contrast, a positive relation between low serum albumin and incident CHD was observed with greater consumption of AA. Notably, these associations were all strengthened after further adjustment for anti-hypertensive medication use, plasma HDL, LDL and triglycerides, systolic blood pressure, BMI and diabetes status. Low serum albumin level (2.0~3.7g/dL) was related to 36% (95%CI=9%-69%, p=0.007) and 44% (95%CI=15%-79%, p=0.001) increased risk of incident CHD among participants with the lowest intake of LA and the highest intake of AA , respectively.

Dietary intake of DHA was also found to significantly modify the albumin-CHD relation (Pinteraction=0.009). However, lower serum albumin level significantly predicted higher risk of incident CHD (β=-0.64, P_{linear}<0.001) among only individuals who consumed moderate amounts of DHA. Similar results were also found for dietary intake of EPA.

As shown in **Figure 3.3.1**, cubic spline analyses were performed in a sub-sample (n=13,509) excluding extreme observations of serum albumin (i.e. <3.2g/dL or >4.5g/dL), which illustrated the above findings.

For incident IS, all examined associations followed the same pattern as with incident CHD (**Table 3.3.3**), whereas only the interaction between dietary intake of omega fatty acids (i.e. EPA+DHA) and serum albumin level reached statistical significance ($P_{interaction}$=0.01). However, after excluding the participants with extreme serum albumin level, the cubic spline analyses revealed no interaction between dietary intake of AA and serum albumin in relation to incident IS (**Figure 3.3.2, Panel B**).

Use a summary score IBRS of levels of VIIIc, WBC, fibrinogen and serum albumin, found no significant results.

Sensitivity analyses

Since fatty acid intake and inflammatory and hemostatic biomarkers were measured at baseline, all the above associations were re-evaluated in the sensitivity analyses, using a shorter follow-up, i.e. CHD and IS incidence tracked through year 1999. A total of 1189 incident CHD events and 333 incident IS events were observed during follow-up. Similar pattern of the above associations was observed, whereas none of the relations reached statistical significance.

To elucidate the unexpected findings of EPA and DHA, we further evaluated the interaction between dietary fish intake (i.e. canned tuna fish, dark meat fish, and other fish) and serum albumin in relation to incident CHD and IS. Separate analyses were performed for tuna fish, dark meat fish, and other fish. Participants were categorized into

three groups according to their dietary intake frequency of each type of fish, i.e. one serving per week or more, 1-3 servings per month, and almost never. As shown in **Table 3.3.4**, among participants who almost never consumed canned tuna fish, low serum albumin level (2.0~3.7g/dL), compared to high serum albumin level (4.0~5.1g/dL), was associated with about 23% (95%CI=3%-47%) higher risk of incident CHD (P_{linear}=0.02), adjusting for baseline age, gender, race-center group, total energy intake, drinking status, smoking status, physical activity and education level. As expected, such association was significantly attenuated with increasing consumption of canned tuna fish ($P_{interaction}$=0.01). However, the findings for dark meat fish and other fish followed the similar pattern observed for EPA and DHA. Finally, similar results were found for the examination of incident IS, although the effect modification of dietary fish intake did not reach statistical significance (data not shown). Additional adjustment for fruit and vegetable intake did not materially change the estimates (data not shown).

3.3.4 Discussion

Fatty acids, inflammation and CVD risk are closely related in a large pool of epidemiological studies. However, few studies have directly examined how dietary fatty acid intake modulates the inflammation-CVD relation. In the current study among middle-aged to old adults, we reported significant interactions between serum albumin level and dietary intakes of LA and AA in relation to incident CHD. Low serum albumin level at baseline predicted high risk of CHD incidence during over 15 years follow-up, which, however, was attenuated with increasing dietary LA intake, but with decreasing

dietary AA intake. Notably, similar patterns were observed in evaluation of incident IS, as well as using shorter follow-up period, despite of less statistical significance.

Serum albumin is the most abundant protein for transporting free fatty acids in blood. As a negative acute-phase protein, serum albumin level can be down-regulated under conditions of malnutrition (especially protein deficiency) and inflammation[34]. Consistently, high levels of other inflammatory/hemostatic biomarkers examined in the current study, i.e. vWF, VIIIc, WBC and fibrinogen, were found to be correlated with low serum albumin level (data not shown). There have been a few studies, including previous ARIC studies, showing that low level, versus appropriate high level, of serum albumin were associated with increasing risk of CVD morbidity and mortality[18, 36, 38, 301].

LA is an essential fatty acid, which can be primarily found in many vegetable oils and accounts for 85%~90% of ω6 PUFA dietary intake[115]. Our findings of potential cardioprotection of LA corroborated a few previous study results[307, 308]. For example, by following a cohort of 1,551 middle-aged men for 15 years, Laaksonen et al. found that increasing levels of LA, as measured in both of diets and serum, were associated with up to about 60% reduced CVD mortality[307]. A monotonically inverse relation between dietary LA intake and risk of CHD was also reported in another prospective study among 78,778 women with 20-year duration[308]. Although limiting evidence is available regarding the health role of LA on IS risk, each 5% increment of serum LA fraction was linked, in a Japanese cohort, to 28% and 34% lower risk of total stroke and IS, respectively[309].

However, concerns have been raised given dietary LA can be metabolized into longer-chain ω6 PUFA in human body, including AA. In contrast to LA, AA has been shown to be a substrate for many pro-inflammatory eicosanoids and thus involved in a variety of pathogenesis processes[116]. Although little population-based evidence revealed the proinflammatory effect of LA, there were *in vitro* studies showing that endothelial cell activation and vascular inflammation may be induced by LA via PI3K/Akt- and ERK1/2-mediated proinflammatory signaling pathways[120]; and under condition of oxidative stress and the stimulus of LA, vascular smooth muscle cells could increase the production of inflammatory chemokine (i.e. IL-8), mediated by AA metabolites[121]. Meanwhile, since the metabolism of LA into longer-chain ω6 PUFA competes with ω3 PUFA (e.g. from α-linolenic acid (ALA) to EPA and DHA) for elongase and desaturase, increasing dietary intake of LA may inhibit the generation of EPA and DHA in human body. Due to the well-established health beneficial role of EPA and DHA[290-295], arguments may sought to reduce dietary LA consumption. However, notably, the natural endogenous conversion of LA to AA is extremely low[119] and none of the AA profiles in serum cholesterol esters or erythrocyte and platelet membranes were found to be significantly altered with variations of total dietary ω6 PUFA intake[310]. In an 8-week double-blinded randomized clinical trial (RCT), the reduction of IL-6 production by fish oil intervention was not different between groups with high (7%kcal) and low (4%kcal) LA intake[98]. Further, increasing evidence has supported that increasing dietary intake of ω6 PUFA (especially LA), even up to 20% of total energy, was associated with lower CVD risk, such as lower LDL, inflammatory or hemostatic biomarkers, or blood pressure[311]. And based on the existing

evidence, an advisory statement issued by the American Heart Association (AHA), which mostly focused on LA, concluded that consuming at least 5%-10% of calories from ω6 PUFA should be recommended[115]. In the present ARIC study, the median LA intakes were 2.78%, 4.10% and 5.83% of total energy across the three distribution groups and therefore, it is not surprising to see that the low serum albumin was on longer associated incident CHD or IS among participants with highest intake of dietary LA (i.e. participants in the $\geq 75^{th}$ percentile distribution).

Interestingly, by contrast to LA and the recommendation of ω6 PUFA consumption, the prediction of low serum albumin on high risk of CHD/IS was strengthened with the increasing dietary intake of AA in the current ARIC population. Such findings met our *priori* hypothesis and may be attributed to the widely proposed, especially in *in vitro* and animal studies, pro-inflammatory characteristics of AA[116]. Evidence did showed that several free radical oxidation products of AA, e.g. F2-isoprostanes, were found to be significantly elevated among patients with angiographic evidence of coronary artery disease[253]. And the 2-week randomized AA intervention (40mg/d, containing an inflammatory fat blend) among 30 healthy adults significantly increased the concentrations of prostaglandin E2 and leukotriene B4, two inflammatory factors[312]. However, inconsistent findings were also widely reported. By comparing 1,998 CHD cases to 6,913 controls in a meta-analysis, Harris et al. demonstrated no relation of CHD risk with AA content in phospholipids-rich tissue, but positive relation with that in adipose tissue[313]. Additionally, both of plasma cholesterol ester and phospholipids AA contents were inversely related to incident CHD and heart failure in a sub-group (in

Minnesota survey center) of current ARIC population[77, 291]. Neverthelss, it is worth noting that the correlations between dietary AA intake and plasma compositions of AA, EPA and DHA were all fairly low (all $r<0.1$, detail data not shown) in that sub-group, although there was evidence showing that dietary AA intake and even low-dose AA supplementation intervention were both positively linked to blood AA composition[314, 315]. Importantly, most research to date, which examined the AA-inflammation or AA-CHD relations, targeted blood AA composition, whereas dietary AA intake can eventually influence the endogenous profile in several tissues (e.g. adipose tissue) in addition to blood, as well as in various metabolites (e.g. free fatty acids). Therefore, further studies are warranted to elucidate and clarify the discrepancies in study findings.

Contrary to our hypothesis, serum albumin level was associated only with incident CHD/IS among the participants with moderate (25^{th}-75^{th} percentile of distribution) dietary intakes of EPA and DHA. In the sensitivity analyses, we found that such findings may be partially attributed to the dietary consumptions of dark meat fish (e.g. salmon, mackerel, swordfish, sardines, bluefish, etc.) and other fish (e.g. cod, perch, catfish, etc.), but not canned tuna fish. Meanwhile, in addition to the types of fish and consumption frequency, the cooking methods that people used may also influence the study results when assessing the health role of EPA and DHA. For example, in a study among four ethnic groups, the plasma phospholipids compositions of EPA and DHA were only positively correlated with nonfried fish intake, but not with nonfried shellfish, fried fish, or fish in mixed dishes; and the correlation found for the nonfried fish intake was further attenuated by taking into account the type of seafood[316]. And among adults aged≥65yr,

only the consumption of tuna or other broiled or baked fish, but not fried fish, was inversely associated with risk of various CVD endpoints[317, 318]. Therefore, different methods for fish processing and preparation may possibly explain the current unexpected findings, which information, however, was not collected at baseline of the ARIC study and it is beyond the scope of the current analyses.

Due to the high correlation of serum albumin with levels of vWF, VIIIc, fribrinogen and WBC, we expected some interactions of dietary fatty acid intake with vWF, VIIIc, fribrinogen and WBC in relation to incident CHD and IS, whereas nothing was found among the current population in this regard. In the previous study among ARIC population, low serum albumin and higher plasma levels of vWF, VIIIc, fribrinogen and WBC were all found to be significantly associated with higher risk of developing CVD events[18]. However, the magnitude of these relations seemed to be stronger for vWF, VIIIc, fribrinogen and WBC than that for serum albumin[18], and thus may be harder to be modified (e.g. enhanced, eliminated or reversed) simply via dietary fatty acid consumption. Especially, the participants in the ARIC study are middle-aged to elderly with generally higher inflammation level due to aging and may be at higher risk of CVD, compared to young population. In addition, the variation of dietary fatty acid intake among the ARIC population was relatively low. Although future studies are warranted to confirm our current findings, such discrepancy between different inflammatory/hemostatic biomarkers may have important implications for public health practice.

One limitation of the current study was that only baseline information of dietary fatty acid intake and inflammation/hemostasis was used, and therefore we were not able to account for the changes of these factors overtime. Fewer number of incident IS events, compared to that of incident CHD, may partially explain the non-significant effect modification of fatty acid intake, whereas we did observe similar pattern of associations for both incident CHD and IS.

In conclusion, we showed in a prospective study with over 15-year follow-up that the association between low serum albumin level and high risk of incident CHD/IS was attenuated with increasing dietary intake of LA, but was enhanced with increasing dietary AA intake. However, the relations between other inflammatory/hemostasis biomarkers, including vWF, VIIIc, fribrinogen and WBC, and incident CHD/IS were not modified by dietary fatty acid consumption. Although more studies should be conducted to elucidate the discrepancy and rule out the possibility of significance resulting from chance, our findings supported the dietary recommendation for consuming LA to a certain extent, whereas reducing AA intake appropriately may also be important.

Table 3.3.1 Participants' characteristics at baseline by groups of incident coronary heart disease (CHD) and ischemic stroke (IS) developed during follow-up through year 2007: ARIC (n=13,734)

	No CHD (n=11,586)	CHD (n=2,148)	No IS (n=13,055)	IS (n=679)
Age (years)	53.8±5.7	55.5±5.5	53.9±5.7	56.3±5.6
Men (%)	39.8	63.2	43.0	51.3
White (%)	74.1	76.9	75.4	58.0
Education level>high school (%)	54.3	59.7	54.7	63.6
Ever-drinker (%)	74.4	77.6	75.1	71.3
Ever-smoker (%)	55.2	68.3	57.0	62.7
Physical active (%, sport index≥2)	72.1	71.0	72.2	67.5
Total energy intake (kcal)	1604±567	1673±595	1614±571	1634±582
BMI (kg/m^2)	27.5±5.4	28.4±5.1	27.6±5.3	28.8±5.5
Systolic blood pressure (mmHg)	120.0±18.2	126.2±19.2	120.4±18.2	131.3±21.6
Plasma HDL-C (mmol/L)	1.4±0.5	1.2±0.4	1.4±0.4	1.3±0.4
Plasma LDL-C (mmol/L)	3.5±1.0	3.9±1.0	3.5±1.0	3.7±1.1
Plasma triglycerides (mmol/L)	1.4±0.9	1.8±1.2	1.5±1.0	1.7±1.2
Anti-hypertensive medication use (%)	26.2	38.3	27.2	46.4
Diabetes (%)	8.7	21.9	9.9	27.5
Fatty acid intake (%kcal)				
16:0 (palmitic acid)	6.59±1.56	6.78±1.56	6.62±1.56	6.68±1.60
16:1 (palmitoleic acid)	0.80±0.23	0.83±0.23	0.80±0.23	0.85±0.24
18:0 (stearic acid)	3.15±0.87	3.26±0.86	3.16±0.87	3.20±0.88
18:1 (oleic acid)	11.46±2.78	11.81±2.76	11.51±2.78	11.69±2.89
18:2ω6 (linoleic acid)	4.27±1.41	4.32±1.45	4.29±1.42	4.14±1.29
18:3ω3 (α-linolenic acid)	0.40±0.09	0.41±0.09	0.40±0.09	0.41±0.09
20:4ω6 (arachidonic acid)	0.078±0.033	0.079±0.032	0.078±0.032	0.084±0.034
20:5ω3 (EPA)	0.055±0.056	0.051±0.051	0.055±0.055	0.056±0.054
22:6ω3 (DHA)	0.109±0.100	0.098±0.092	0.107±0.099	0.109±0.095

LC-ω3 (EPA+DHA)	0.149±0.152	0.135±0.139	0.147±0.150	0.150±0.145
Inflammatory and hemostatic biomarkers				
vWF (%)	116±47	124±50	116±47	137±64
Factor VIIIc (%)	130±39	136±43	130±39	146±49
WBC (x1000 cells/mm^2)	6.0±1.9	6.5±2.1	6.1±1.9	6.5±2.2
Fibrinogen (mg/L)	300±64	315±66	301±64	322±73
Albumin (g/dL)	3.9±0.3	3.9±0.3	3.9±0.3	3.8±0.3

Table 3.3.2 Hazard ratio (95% CI) of incident CHD in relation to serum albumin, stratified by fatty acid intake categories: ARIC CHD [a] (n=13,734)

Fatty acid intake categories		Albumin tertiles 2.0~3.7g/dL	3.8~3.9g/dL	4.0~5.1g/dL	β	P_{linear}
18:2 (P_{interx}=0.04 [b]*)*						
≤25th percentile	HR of Model 1[c]	1.29 (1.05, 1.60)	0.92 (0.74, 1.14)	1	-0.53	0.02
Median=2.78%kcal	HR of Model 2[d]	1.36 (1.09, 1.69)	1.00 (0.80, 1.24)	1	-0.62	0.007
25th-75th percentile	HR of Model 1	1.17 (1.01, 1.36)	1.02 (0.88, 1.18)	1	-0.32	0.04
Median=4.10%kcal	HR of Model 2	1.31 (1.12, 1.54)	1.10 (0.94, 1.27)	1	-0.55	<0.001
≥75th percentile	HR of Model 1	1.05 (0.85, 1.29)	0.85 (0.69, 1.04)	1	-0.10	0.63
Median=5.83%kcal	HR of Model 2	1.07 (0.86, 1.32)	0.87 (0.70, 1.08)	1	-0.13	0.55
20:4 (P_{interx}= 0.05)						
≤25th percentile	HR of Model 1[c]	0.97 (0.78, 1.21)	0.77 (0.62, 0.96)	1	0.08	0.73
Median=0.045%kcal	HR of Model 2[d]	1.00 (0.80, 1.26)	0.82 (0.66, 1.02)	1	0.01	0.98
25th-75th percentile	HR of Model 1	1.21 (1.04, 1.40)	1.02 (0.88, 1.18)	1	-0.38	0.01
Median=0.073%kcal	HR of Model 2	1.32 (1.14, 1.54)	1.10 (0.95, 1.28)	1	-0.56	<0.001
≥75th percentile	HR of Model 1	1.30 (1.06, 1.60)	1.02 (0.83, 1.25)	1	-0.54	0.01
Median=0.113%kcal	HR of Model 2	1.44 (1.15, 1.79)	1.09 (0.87, 1.35)	1	-0.74	0.001
20:5 (P_{interx}= 0.07)						
≤25th percentile	HR of Model 1[c]	1.06 (0.87, 1.30)	0.93 (0.76, 1.13)	1	-0.12	0.56
Median=0.010%kcal	HR of Model 2[d]	1.21 (0.98, 1.49)	1.06 (0.87, 1.30)	1	-0.38	0.08
25th-75th percentile	HR of Model 1	1.29 (1.11, 1.50)	0.93 (0.80, 1.08)	1	-0.54	<0.001
Median=0.040%kcal	HR of Model 2	1.33 (1.14, 1.55)	0.96 (0.83, 1.12)	1	-0.59	<0.001
≥75th percentile	HR of Model 1	1.05 (0.84, 1.31)	1.01 (0.82, 1.25)	1	-0.09	0.70

Median=0.106%kcal	HR of Model 2	1.13 (0.90, 1.43)	1.05 (0.84, 1.30)	1	-0.25	0.29
22:6 (P_{interx}= 0.009)						
≤25th percentile	HR of Model 1[c]	0.97 (0.79, 1.18)	0.94 (0.78, 1.14)	1	0.07	0.74
Median=0.026%kcal	HR of Model 2[d]	1.06 (0.86, 1.32)	1.05 (0.86, 1.28)	1	-0.12	0.56
25th-75th percentile	HR of Model 1	1.35 (1.17, 1.57)	0.93 (0.80, 1.09)	1	-0.64	<0.001
Median=0.079%kcal	HR of Model 2	1.41 (1.21, 1.64)	0.98 (0.84, 1.14)	1	-0.71	<0.001
≥75th percentile	HR of Model 1	1.03 (0.82, 1.30)	1.00 (0.81, 1.24)	1	-0.06	0.80
Median=0.204%kcal	HR of Model 2	1.15 (0.91, 1.46)	1.03 (0.83, 1.29)	1	-0.28	0.24
Omega (P_{interx}= 0.08)						
≤25th percentile	HR of Model 1[c]	0.99 (0.81, 1.21)	0.92 (0.75, 1.11)	1	0.02	0.94
Median=0.028%kcal	HR of Model 2[d]	1.13 (0.92, 1.40)	1.04 (0.85, 1.27)	1	-0.25	0.25
25th-75th percentile	HR of Model 1	1.32 (1.14, 1.53)	0.95 (0.82, 1.10)	1	-0.58	<0.001
Median=0.104%kcal	HR of Model 2	1.35 (1.16, 1.57)	0.98 (0.84, 1.15)	1	-0.62	<0.001
≥75th percentile	HR of Model 1	1.06 (0.84, 1.33)	1.00 (0.80, 1.23)	1	-0.11	0.63
Median=0.293%kcal	HR of Model 2	1.16 (0.91, 1.46)	1.02 (0.82, 1.28)	1	-0.29	0.23

[a] Categories are ≤25th , 25th -75th , and ≥75th percentile of fatty acid intake distribution;

[b] p-value for the interaction between fatty acid intake and serum albumin (tested in Model 1)

[c] Model 1: adjusted for baseline age, gender, race-center group, total energy intake, drinking status, smoking status, physical activity and education level.

[d] Model 2: Model 1+ further adjusted for anti-hypertensive medication use, plasma HDL, LDL and triglycerides, systolic blood pressure, BMI and diabetes status.

Table 3.3.3 Hazard ratio (95% CI) of incident IS in relation to serum albumin, stratified by fatty acid intake categories: ARIC IS [a] (n=13,734)

Fatty acid intake categories		Albumin tertiles 2.0~3.7g/dL	3.8~3.9g/dL	4.0~5.1g/dL	β	P_{linear}
18:2 (P_{interx}=0.18 [b])						
≤25th percentile	HR of Model 1[c]	2.08 (1.44, 3.01)	1.00 (0.66, 1.53)	1	-1.60	<0.001
Median=2.78%kcal	HR of Model 2[d]	2.39 (1.62, 3.54)	1.17 (0.76, 1.79)	1	-1.79	<0.001
25th-75th percentile	HR of Model 1	1.34 (1.04, 1.72)	1.05 (0.80, 1.36)	1	-0.60	0.02
Median=4.10%kcal	HR of Model 2	1.41 (1.08, 1.84)	1.09 (0.83, 1.43)	1	-0.71	0.009
≥75th percentile	HR of Model 1	1.23 (0.83, 1.82)	0.86 (0.56, 1.31)	1	-0.46	0.26
Median=5.83%kcal	HR of Model 2	1.45 (0.95, 2.19)	1.02 (0.67, 1.57)	1	-0.81	0.06
20:4 (P_{interx}= 0.58)						
≤25th percentile	HR of Model 1[c]	1.20 (0.80, 1.81)	0.72 (0.46, 1.12)	1	-0.42	0.34
Median=0.045%kcal	HR of Model 2[d]	1.37 (0.89, 2.12)	0.76 (0.48, 1.20)	1	-0.64	0.17
25th-75th percentile	HR of Model 1	1.55 (1.19, 2.02)	1.04 (0.78, 1.38)	1	-0.93	<0.001
Median=0.073%kcal	HR of Model 2	1.73 (1.31, 2.29)	1.17 (0.87, 1.56)	1	-1.13	<0.001
≥75th percentile	HR of Model 1	1.57 (1.13, 2.18)	1.17 (0.83, 1.67)	1	-0.91	0.006
Median=0.113%kcal	HR of Model 2	1.67 (1.18, 2.37)	1.28 (0.89, 1.84)	1	-1.01	0.004
20:5 (P_{interx}= 0.15)						
≤25th percentile	HR of Model 1[c]	1.28 (0.87, 1.87)	0.85 (0.57, 1.27)	1	-0.56	0.16
Median=0.010%kcal	HR of Model 2[d]	1.52 (1.02, 2.26)	0.96 (0.63, 1.45)	1	-0.89	0.03
25th-75th percentile	HR of Model 1	1.58 (1.23, 2.05)	0.99 (0.75, 1.31)	1	-0.99	<0.001
Median=0.040%kcal	HR of Model 2	1.67 (1.27, 2.20)	1.07 (0.80, 1.42)	1	-1.09	<0.001
≥75th percentile	HR of Model 1	1.43 (0.99, 2.07)	1.19 (0.82, 1.73)	1	-0.71	0.06

Median=0.106%kcal	HR of Model 2	1.61 (1.09, 2.38)	1.36 (0.92, 2.00)	1	-0.91	0.02
22:6 (P_{interx}= 0.13)						
≤25th percentile	HR of Model 1[c]	1.34 (0.93, 1.94)	0.75 (0.50, 1.13)	1	-0.69	0.08
Median=0.026%kcal	HR of Model 2[d]	1.49 (1.01, 2.19)	0.80 (0.52, 1.21)	1	-0.88	0.03
25th-75th percentile	HR of Model 1	1.60 (1.23, 2.07)	1.00 (0.75, 1.33)	1	-1.01	<0.001
Median=0.079%kcal	HR of Model 2	1.75 (1.33, 2.31)	1.10 (0.82, 1.48)	1	-1.18	<0.001
≥75th percentile	HR of Model 1	1.32 (0.91, 1.91)	1.25 (0.87, 1.79)	1	-0.52	0.16
Median=0.204%kcal	HR of Model 2	1.50 (1.01, 2.21)	1.41 (0.97, 2.05)	1	-0.73	0.06
Omega (P_{interx}= 0.01)						
≤25th percentile	HR of Model 1[c]	1.24 (0.85, 1.80)	0.84 (0.56, 1.26)	1	-0.49	0.21
Median=0.028%kcal	HR of Model 2[d]	1.40 (0.94, 2.08)	0.91 (0.60, 1.38)	1	-0.74	0.07
25th-75th percentile	HR of Model 1	1.68 (1.30, 2.17)	0.98 (0.74, 1.31)	1	-1.12	<0.001
Median=0.104%kcal	HR of Model 2	1.83 (1.39, 2.40)	1.08 (0.80, 1.45)	1	-1.28	<0.001
≥75th percentile	HR of Model 1	1.29 (0.89, 1.88)	1.22 (0.84, 1.75)	1	-0.49	0.19
Median=0.293%kcal	HR of Model 2	1.42 (0.95, 2.10)	1.37 (0.94, 2.00)	1	-0.64	0.10

[a] Categories are ≤25th , 25th -75th , and ≥75th percentile of fatty acid intake distribution;

[b] p-value for the interaction between fatty acid intake and serum albumin (tested in Model 1)

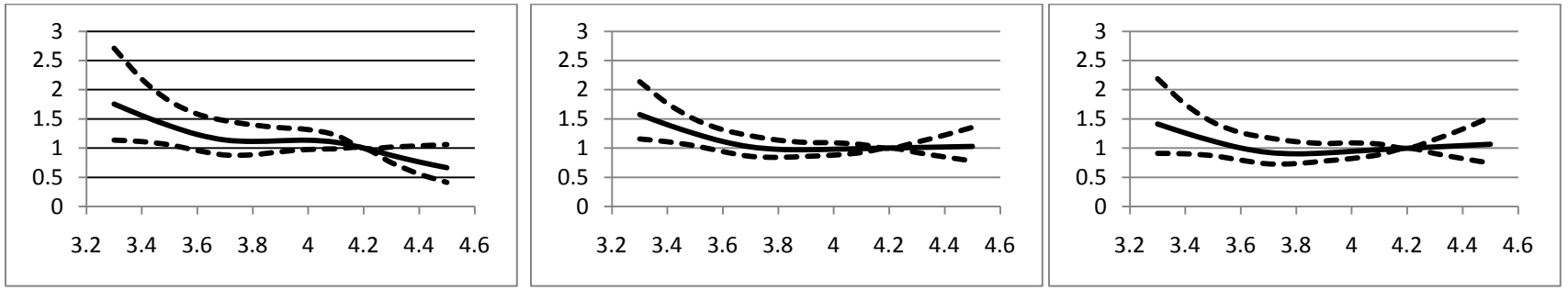
[c] Model 1: adjusted for baseline age, gender, race-center group, total energy intake, drinking status, smoking status, physical activity and education level.

[d]Model 2: Model 1+ further adjusted for anti-hypertensive medication use, hormone replacement therapy use, plasma HDL, LDL and triglycerides, systolic blood pressure, BMI and diabetes status.

Figure 3.3.1 Cubic spline analysis: HR of CHD in relation to serum albumin by fatty acid intake categoriesa, adjusting for age, gender, race-center, total energy intake, drinking status, smoking status, physical activity and education level: ARIC CHD.

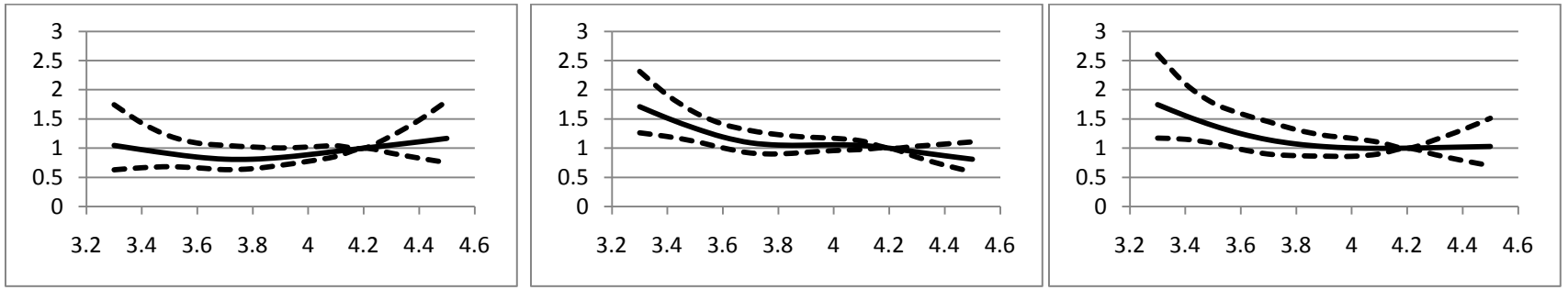
Panel A. Linoleic acid (18:2, LA)

Category 1 Category 2 Category 3



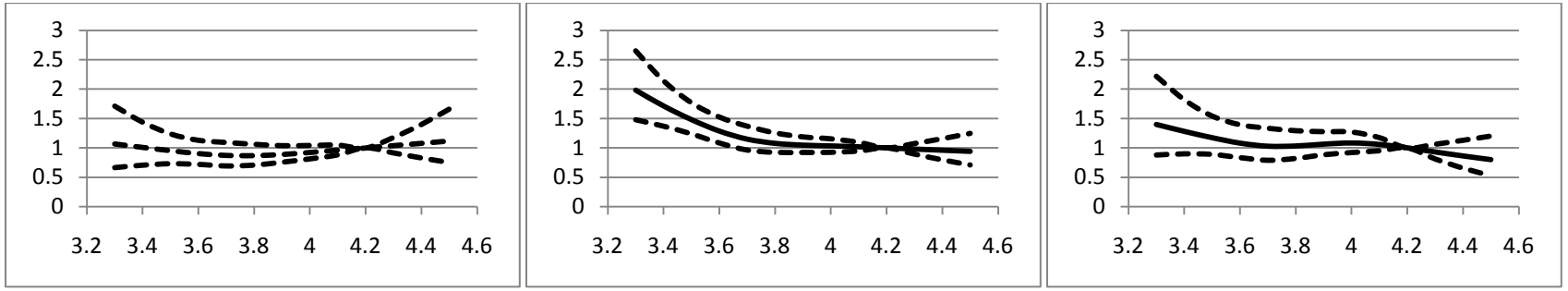
Panel B. Arachidonic acid (20:4, AA)

Category 1 Category 2 Category 3



Panel C. DHA (22:6)

Category 1 Category 2 Category 3

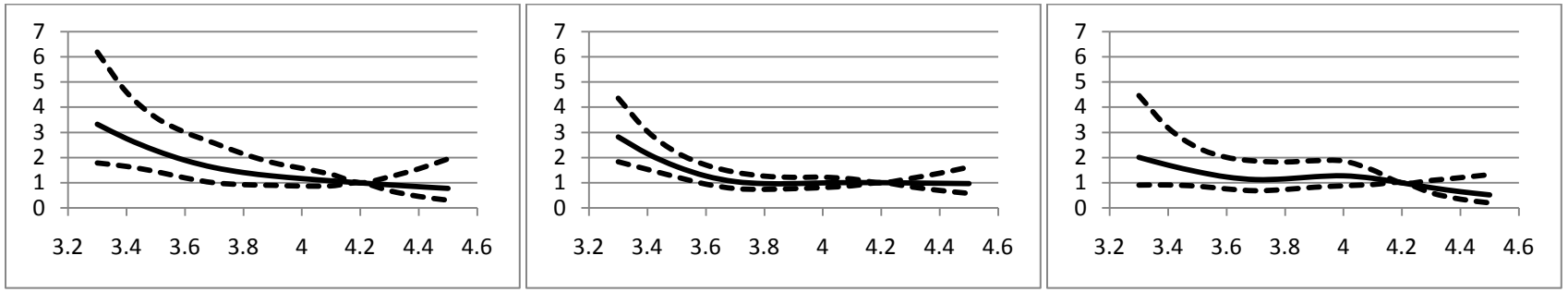


[a] The solid line represents the HR; dotted lines are 95%CIs; sub-samples were used for the cubic spline analyses by excluding participants with extreme levels of serum albumin. Knots were located at the 5th , 25th , 75th and 95th percentile of the albumin distribution. The reference value was set at the 90th percentile of the serum albumin distribution; Categories 1-3 are ≤25th , 25th -75th and ≥75th percentile of fatty acid intake distribution, respectively.

Figure 3.3.2 Cubic spline analysis: HR of IS in relation to serum albumin by fatty acid intake categoriesa, adjusting for age, gender, race-center, total energy intake, drinking status, smoking status, physical activity and education level: ARIC IS.

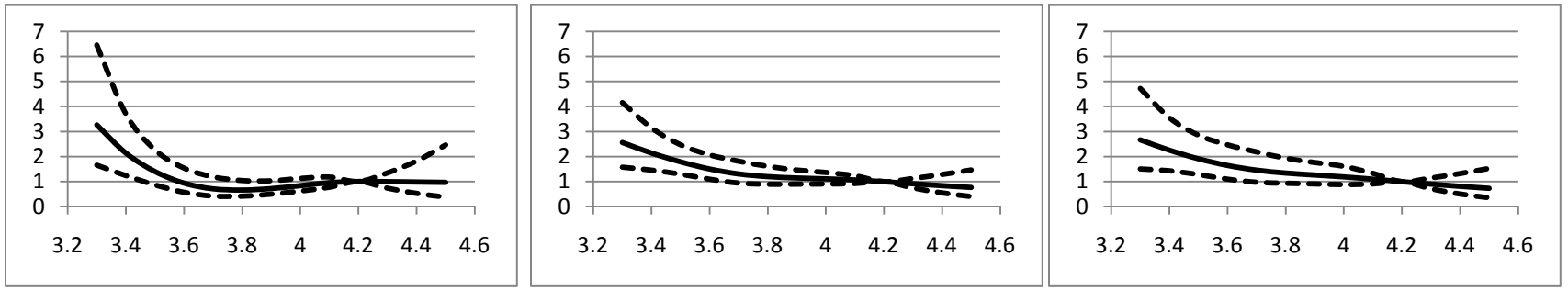
Panel A. Linoleic acid (18:2, LA)

Category 1 Category 2 Category 3



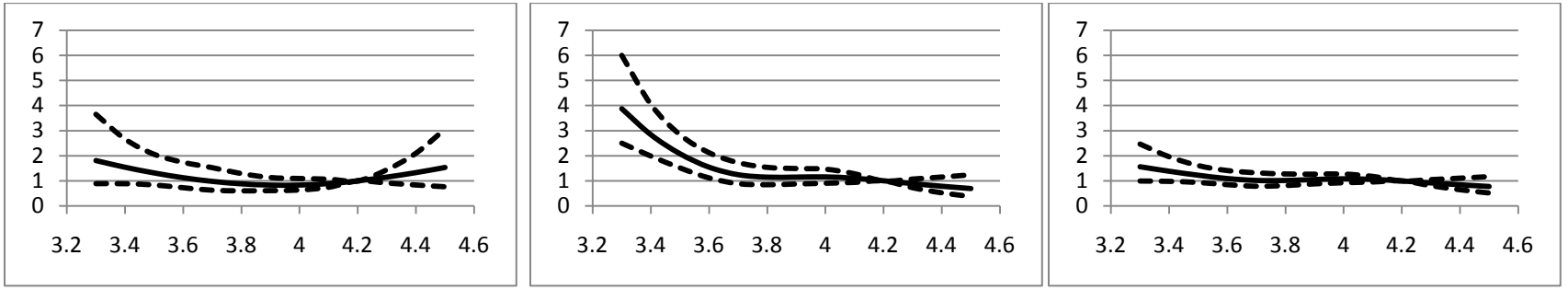
Panel B. Arachidonic acid (20:4, AA)

Category 1 Category 2 Category 3



Panel C. Omega (EPA+DHA)

Category 1 Category 2 Category 3



[a] The solid line represents the HR; dotted lines are 95%CIs; sub-samples were used for the cubic spline analyses by excluding participants with extreme levels of serum albumin.
Knots were located at the 5^{th} , 25^{th} , 75^{th} and 95^{th} percentile of the albumin distribution. The reference value was set at the 90^{th} percentile of the serum albumin distribution; Categories 1-3 are $\leq 25^{th}$, 25^{th} -75^{th} and $\geq 75^{th}$ percentile of fatty acid intake distribution, respectively.

Table 3.3.4 Hazard ratio (95% CI) of incident CHD in relation to serum albumin, stratified by fish intake categories: ARIC CHD [a] (n=13,927)

Fish intake categories		Albumin tertiles 2.0~3.7g/dL	3.8~3.9g/dL	4.0~5.1g/dL	β	P_{linear}
Tuna (P_{interx}=0.01 [b])						
Almost never	HR of Model 1[c]	1.23 (1.03, 1.47)	1.04 (0.87, 1.24)	1	-0.42	0.02
	HR of Model 2[d]	1.31 (1.09, 1.57)	1.13 (0.95, 1.36)	1	-0.53	0.004
1-3 per month	HR of Model 1	1.11 (0.93, 1.32)	0.82 (0.69, 0.99)	1	-0.21	0.27
	HR of Model 2	1.23 (1.02, 1.48)	0.92 (0.76, 1.11)	1	-0.42	0.03
≥1 per week	HR of Model 1	1.16 (0.96, 1.40)	1.02 (0.85, 1.22)	1	-0.30	0.12
	HR of Model 2	1.22 (1.00, 1.49)	0.99 (0.82, 1.19)	1	-0.40	0.05
Dark (P_{interx}= 0.01)						
Almost never	HR of Model 1[c]	1.13 (0.98, 1.29)	0.87 (0.76, 1.00)	1	-0.26	0.07
	HR of Model 2[d]	1.22 (1.06, 1.41)	0.93 (0.81, 1.07)	1	-0.41	0.006
1-3 per month	HR of Model 1	1.38 (1.13, 1.68)	1.06 (0.87, 1.30)	1	-0.65	0.001
	HR of Model 2	1.46 (1.19, 1.81)	1.11 (0.91, 1.37)	1	-0.77	<0.001
≥1 per week	HR of Model 1	0.94 (0.71, 1.23)	1.09 (0.86, 1.39)	1	0.12	0.66
	HR of Model 2	0.99 (0.74, 1.32)	1.13 (0.88, 1.46)	1	0.02	0.94
Other (P_{interx}= 0.10)						
Almost never	HR of Model 1[c]	1.04 (0.87, 1.25)	0.84 (0.70, 1.00)	1	-0.08	0.66
	HR of Model 2[d]	1.15 (0.94, 1.39)	0.95 (0.79, 1.14)	1	-0.27	0.17
1-3 per month	HR of Model 1	1.32 (1.10, 1.60)	1.11 (0.92, 1.34)	1	-0.56	0.004
	HR of Model 2	1.39 (1.14, 1.70)	1.17 (0.97, 1.42)	1	-0.66	0.001
≥1 per week	HR of Model 1	1.15 (0.97, 1.37)	0.96 (0.81, 1.14)	1	-0.29	0.09

	HR of Model 2	1.23 (1.03, 1.47)	0.98 (0.82, 1.16)	1	-0.42	0.02

[a] Tuna=Canned tuna fish; Dark=Dark meat fish; Other=Other fish;
Categories are one serving per week or more, 1-3 servings per month, and almost never.
[b] p-value for the interaction between fatty acid intake and serum albumin (tested in Model 1)
[c] Model 1: adjusted for baseline age, gender, race-center group, total energy intake, drinking status, smoking status, physical activity and education level.
[d]Model 2: Model 1+ further adjusted for anti-hypertensive medication use, plasma HDL, LDL and triglycerides, systolic blood pressure, BMI and diabetes status.

3.4 Manuscript 4 – Inflammation and hemostasis mediates the association of plasma phospholipids fatty acids with coronary heart disease and ischemic stroke incidence: The Atherosclerosis Risk in Communities (ARIC) study

From molecular perspective, fatty acids have been broadly proposed may influence risk of cardiovascular disease (CVD) via promoting or preventing inflammation and hemostatic dysfunction. However, this has not been directly examined among a free-living population. This study aimed to explore, whether and to what extent, inflammation and hemostasis mediated the relation of fatty acids, as measured in plasma phospholipids (PL), with incident coronary heart disease (CHD) and ischemic stroke (IS) in a sub-cohort (n=4,009) enrolled in a large prospective study, the Atherosclerosis Risk in Communities (ARIC) study. Inflammatory/hemostatic biomarkers and PL fatty acid profile were measured at baseline (year 1987-89), while CVD events were identified through year 2007. After exclusions, a total of 3,715 participants were included in the current analysis. During over 15-year follow-up, 532 and 145 participants developed incident CHD and IS, respectively. After controlling for possible confounders, inflammation and hemostasis, reflected by levels of factor VIIIc (VIIIc), white blood cell count (WBC) and fibrinogen, completely mediated the positive relation of PL 20:3ω6 fatty acid with incident CHD. A similar, but weaker, mediation effect of inflammation/hemostasis was found for PL 18:0 fatty acid in relation to incident CHD, and PL 16:1ω7 fatty acid in relation to incident IS. However, in contrast, baseline PL

components of 17:0 and 20:4ω6 fatty acids were inversely associated with risk of incident CHD, which was partially explained by lower WBC, but not VIIIc or fibrinogen. Inflammation and hemostasis contribute, in different magnitudes, to the diverse associations between individual fatty acids and CVD risk. The pathophysiology of CHD and IS, despite sharing a few common risk factors, may differ to some extent.

3.4.1 Introduction

Blood levels of fatty acids, reflecting the dietary fatty acids intake, have been associated with cardiovascular diseases (CVD) and its risk factors, including inflammation[77, 191, 284, 291, 319, 320]. The inflammatory pathology begins with the damage of vascular smooth muscle by circulating oxidized fat components and is closely interwined with a series of reactions on vascular endothelium, such as endothelial and hemostatic dysfunction. Although initially serving as a protective mechanism against the damage, prolong inflammation and hemostatic dysfunction can be detrimental, in which macrophages are recruited to the lesion site, releasing proinflammatory cytokines and promoting the formation of plaques on vascular walls[16, 20]. The thickening of arterial walls may eventually lead to incident coronary heart disease (CHD), as well as ischemic stroke (IS)[278]. A few epidemiological studies have shown significant associations between CVD outcomes and levels of circulating inflammatory/hemostatic biomarkers, such as factor VIIIc, fibrinogen and white blood cell counts (WBC), C-reactive protein etc.[16, 18, 37, 203, 275, 277, 280-282].

Fatty acids are actively involved in the progression of inflammation and cardiovascular diseases (CVD), which have been examined in a few studies[283-285].

Different types of fatty acids may exert their health effects by modulating the pro- or anti-inflammatory markers[59, 286-288]. Generally, saturated fatty acids (SFA) are pro-inflammatory, resulting in increased risk of CHD and IS[40, 238, 289]. However, stearic acid (18:0) may not be as pro-atherogenic as palmitic acid (16:0)[81]; pentadecanoic acid (15:0) and heptadecanoic acid (17:0), specifically found in ruminant animal products (e.g. dairy), have been linked to reduced CVD risk[89, 211, 216, 217]. Relatively consistent evidence has been shown regarding the anti-inflammatory and cardioprotective effects of ω3 polyunsaturated fatty acids (PUFA), especially eicosapentaenoic acid (20:5ω3, EPA) and docosahexaenoic acid (22:6ω3, DHA)[290-295]. In contrast, no consistent evidence is available to conclude the health roles of monounsaturated fatty acids (MUFA) and ω6 PUFA[210]. For example, although arachidonic acid (AA, 20:4ω6) has been commonly linked to the promotion of inflammation due to its metabolites--eicosanoids[116], its anti-inflammatory effects were also reported[122-124, 321]. Similarly, some but not all studies supported that linoleic acid (18:2ω6) and dihomo-γ-linolenic acid (20:3ω6) may be associated with lower inflammation and CVD risk[91, 93, 291, 296, 321], especially given that they are the substrates for endogenous production of 20:4ω6 fatty acid.

Compared to the relatively well-established relations of fatty acid with CHD, the evidence on the associations between fatty acids and IS is limited and inconsistent[72-74]. Among 79,839 women who were followed up for 14 years, higher consumption of fish and ω3 PUFA was associated with a reduced risk of thrombotic infarction (i.e. a subtype of IS)[297]. Nevertheless, in a six-month randomized clinical trial conducted in 258 adults ages 45-70 years, Sanders et al. did not observe any difference in levels of IS-related

hemostatic factors among study participants randomized to one of four diets with varying ω6/ω 3 fatty acid ratios[76]. In the Framingham Heart Study, higher intakes of total fat and saturated fatty acid (%kcal) were found to be associated with lower risk of IS in males[75]. Assuming that IS shares similar pathological mechanisms with CHD (e.g. inflammation and hemostasis), and since dietary intake is an important modifiable risk factor for CVD[298], a better understanding of the relations between dietary fatty acids, hemostatic and inflammatory biomarkers, and CHD/IS is warranted.

It has been widely proposed that fatty acids may influence CVD risk via promoting or preventing inflammation and hemostatic dysfunction[59, 286-28840]

(Hall 2009). However, there is no study, so far, directly examining the mediation effect of inflammation/hemostasis on the associations between fatty acids and incident CHD or IS in a free-living population. Therefore, the current study aimed to explore whether and how inflammation and hemostasis mediate the relation of fatty acids, as measured in plasma phospholipids (PL), with incident CHD/IS in a large prospective cohort study, the Atherosclerosis Risk in Communities (ARIC) study. Several previous ARIC studies have related PL fatty acid composition and inflammation and hemostasis, respectively, to CVD outcomes[18, 77, 191, 203, 277, 291, 304]. Based on those evidence, we hypothesized that increased proportions of PL fatty acids, including 16:0, 18:0, 16:1ω7, 18:3ω6 and 20:3ω6, would be associated with higher risk of CHD/IS incidence via higher levels of inflammatory/hemostatic biomarkers, whereas other fatty acids, such as 15:0, 17:0, 18:1ω9, 18:2ω6, 18:3ω3, 20:4ω6, 20:5ω3 and 22:6ω3 would be inversely related to CHD/IS incidence via lower levels of inflammation/hemostatic biomarkers.

3.4.2 Methods

Study population

The Atherosclerosis Risk in Communities (ARIC) study is a prospective cohort study conducted to study subclinical and clinical atherosclerosis and CVD risk factors in middle-aged adults. Four study centers are involved, which are Forsyth County, NC; the city of Jackson, MS; selected suburbs of Minneapolis; and Washington County, MD.

The baseline visit was conducted in 1987-1989, enrolling 15,792 participants. As described previously[187], all protocols were approved by local institutional reviewed boards and data were collected through clinical examination and interviews. Informed consent was sent to all participants. Three additional examinations were performed every 3 years after the baseline visit, with annual follow-up contacts thereafter.

The current study only included 4,009 participants in the Minnesota center whose plasma fatty acid components were saved and analyzed at baseline.

Measurement of inflammatory and hemostatic biomarkers

Fasting blood specimen were drawn from ARIC participants' antecubital vein, from which the serum, plasma and whole blood samples were prepared and stored by standardized protocols in the ARIC hemostasis laboratory[199, 299]. Detailed information on the measurements of inflammatory and hemostatic biomarkers has been reported elsewhere[299, 300]. For the current study, three inflammatory and hemostatic biomarkers were used, including VIIIc, WBC and fibrinogen. WBC was determined in the whole blood sample using a Coulter Counter. Plasma levels of fibrinogen and VIIIc were determined by using the thrombin time titration method, ELISA kits and clotting assays,

respectively[300]. The VIIIc level were then expressed as percent activity by relating the clotting time to a calibration curve constructed for each batch of samples..

Reliability of these measurements of inflammatory and hemostatic biomarkers was tested in subsamples of ARIC participants[302-304]. The reliability coefficients for fibrinogen, VIIIc, and WBC were 0.72, 0.86, and greater than 0.96, respectively; whereas the intra- and interassay variability was all lower than 5%[18, 302-304].

Measurement of plasma phospholipids fatty acids

Blood collection was under standardized conditions for the ARIC study[190]. Fasting blood was collected into 10ml vacuum tubes containing EDTA and sent by courier within 3h to the University of Minnesota Hospital and Clinic Laboratory. The blood was centrifuged at 800×g for 10min at 4℃. Plasma was then separated and dispensed into two 1.5ml aliquots and frozen at -70℃until analyzed for fatty acid content by a single technician.

A detail description of the methods used to analyze plasma fatty acids was published previously[9, 191]. After thawing, 0.5mL plasma was extracted with 0.5mL methanol followed by 1.0mL chloroform under a nitrogen atmosphere. The lipid extract was filtered to remove protein. The PL fractions were separated by thin-layer chromatography with a silica gel plate (Silica Gel H; Analtech, Newark, DE) and 2-stage mobile phase development, which consisted of solvents of petroleum ether, diethyl ether, and glacial acetic acid in ratios of 80:20:1 (by vol) and 40:60:1 (by vol), respectively. The plate was dried between development solvents and the second mobile phase was allowed to migrate for only one-half of the plate length. After re-drying, one lane was sprayed with

dichlorofluorescein to visualize the PL, triglyceride, and free fatty acid bands under ultraviolet light. The PL bands were scraped into separate test tubes, and the lipids were converted to methyl esters of fatty acids by boron trifluoride catalysis. The methyl esters were then separated and measured on a Hewlett-Packard 5890 gas chromatograph (Hewlett-Packard, Avondale, PA) equipped with a 50m FFAP WCOT glass capillary column (J&W Scientific, Folsom, CA) and a flame ionization detector. The identity of each fatty acid peak was ascertained by comparison of the peak's retention time with the retention times of fatty acids in synthetic standard of known fatty acid composition. Each fatty acid (% of total PL fatty acid) was quantified by integrating the area under the peak and dividing the results by the total area for all PL fatty acids. To minimize transcription errors, the data from the gas chromatogram was electronically transferred to a VAX computer (Digital Equipment Corporation, Minneapolis) for data analysis. Test-retest reliability coefficients (individuals sampled 3 times, 2 weeks apart) for various plasma fatty acids ranged from 0.31 to 0.89 for PL [8].

Measurement of other baseline covariates

Baseline information of demographics, medical history, medication use and lifestyle habits, e.g. smoking, physical activity (Baecke 'sport index score'[192]), was obtained via trained interviewers administered questionnaires. Baseline dietary intake was assessed using an interviewer-administered 66-item food frequency questionnaire (FFQ) slightly modified from the version developed and validated by Willett et al.[188], from which the daily nutrient values and total energy intake were calculated for each participant[188]).

Alcohol intake was assessed by asking participants whether they currently drank alcoholic beverages, and, if not, whether they ever had drunk alcohol.

Body mass index (BMI, kg/m^2) was calculated using measurements of weight and height. Sitting blood pressure was assessed three times on each participant after a 5min rest using a random-zero sphygmomanometer; the 2nd and 3rd readings were averaged for the analyses[195]. Hypertension was defined as a systolic blood pressure ≥140mm Hg or a diastolic blood pressure ≥90mm Hg or current use of antihypertensive medications. Fasting blood samples were used for lipid and glucose measurements. Plasma total cholesterol and triglycerides were measured by enzymatic methods[193], and high-density lipoprotein (HDL) cholesterol[176] was measured after dextran-magnesium precipitation. Low density lipoprotein cholesterol (LDL) was calculated using the Friedewald equation[193]. A hexokinase/glucose-6-phosphate dehydrogenase method was used to measure serum glucose and prevalent diabetes mellitus was identified with a serum glucose of ≥126mg/dL (fasting), of ≥200mg/dL (nonfasting), and/or a history of or treatment for diabetes. Prevalent CHD was defined at baseline as a (1) self-reported history of physician diagnosed heart attack; (2) previous MI by ECG; (3) history of coronary bypass surgery; or (4) history of coronary angioplasty.

Assessment of incident CHD and IS

ARIC investigators followed the cohort and ascertained CHD and IS events using standardized methods described previously[198, 199]. Briefly, participants were contacted annually to identify hospitalizations and deaths during the prior year, and trained abstractors surveyed discharge lists from local hospitals and death certificates from state

vital statistics offices for potential cardiovascular and cerebrovascular events. Incident cases of CHD were defined as (1) a definite or probable myocardial infarction (MI); (2) a silent MI; (3) a definite CHD death; or (4) a coronary revascularization[201]. Detailed information on the ascertainment and classification of stroke can be found elsewhere [305]. Strokes were diagnosed based on the criteria adapted from the National Survey of Stroke and incident IS was indentified according to the evidence of acute infarction or no hemorrhage revealed by a brain photocopied neuroimaging (CT or MRI)[203, 305]. Incident cases of CHD and IS were tracked though year 2007.

Statistical analysis

All analyses were performed with SAS (version 9.2, SAS Institute Inc, Cary, NC). Participants were excluded from the analyses if they had prevalent CHD (n=182) and stroke (n=43) at baseline; were non-white (n=37, due to small numbers); were missing plasma PL fatty acid measurements (n=74); were missing measurements of VIIIc (n=3), WBC (n=3) and fibrinogen (n=3); After all the exclusions, a total of 3,715 participants were included in the current study. The following analyses were conducted separately for CHD and IS.

Variables with skewed distribution were log-transformed before entering the analyses and back-transformed to present as appropriate. PL fatty acid components were expressed as % total PL fatty acids. Both of continuous and categorical (quintiles) PL fatty acid variables were used in the analyses. A summary biomarker risk score (IBRS) was also created to combine information on levels of VIIIc, WBC and fibrinogen. For each participant, the values for each biomarker were expressed as a Z-score and the IBRS was

calculated by summing up the three Z-scores[306]: IBRS=(Z_{VIIIc}+Z_{WBC}+$Z_{fibrinogen}$)/3. IBRS were treated as either continuous or categorical (quintiles) variable in the analyses.

Cox proportional hazards models were used to estimate and test the mediation effects of IBRS on the relations between PL fatty acids and incident CHD or IS. Potential confounders were adjusted in models, including continuous variables: age and total energy intake; and categorical variables: sex (men vs. women), drinking status (never-drinker vs. ever-drinker), smoking status (never-smoker vs. ever-smoker), physical activity (sport index score<2 vs. ≥2) and education level (more than high school vs. high school or less).

The mediation effects of IBRS were evaluated by[158, 322-324]: (1) testing the relation between incident CHD (or IS) and IBRS; (2) testing the relation between PL fatty acids and risk of incident CHD (or IS). (3) testing the relation between PL fatty acids and IBRS in generalized linear regression models; If one or more of the relations in steps (1) to (3) were non-significant, we concluded that mediation was not possible or likely and stopped the examination of mediation. Otherwise, the analyses were continued onto the following steps: (4) conducting a multiple regression analysis by simultaneously including PL fatty acids and IBRS in the model to predict CHD (or IS) incidence; (5) the mediation effect of IBRS was calculated by subtracting the regression coefficient of PL fatty acids in (4) from the coefficient of PL fatty acids in (2).

In step (1) to (3), categorical predictors of PL fatty acids and IBRS were used first in order to better clarify the association patterns. Linear trends and the weighted average slopes (β coefficient) of dependent variables across quintiles of predictors were tested and

generated by using the median of predictors by each category. However, the continuous PL fatty acids and IBRS were used to obtain the regression slopes (β coefficient) and p-values for the associations, which were used in the steps (4) and (5) thereafter.

In order to conclude that a mediation effect is present, the coefficient of PL fatty acids in step (4) should be attenuated than that in step (2). For Cox proportional hazards models, testing the significance of the coefficient for the mediation effect calculated in (5) is equivalent to testing the statistical significance of the coefficient of IBRS in (4)[325].

To evaluate a potential gender difference, we also included multiplicative interaction terms in models 1 as appropriate, i.e. gender x fatty acid or biomarker, which, however, were not statistically significant. Therefore, all analyses were done among the whole population.

In addition to the summary score IBRS, VIIIc, WBC and fibrinogen were also tested individually following the above procedures.

In the sensitivity analyses, since PL fatty acid and inflammatory/hemostatic biomarkers were measured at baseline, all the above associations were first re-evaluated using a shorter follow-up, i.e. CHD and IS incidence tracked through year 1999. To further explore related biological mechanisms, the analyses were also re-conducted by further controlling for continuous variables: BMI, systolic blood pressure, plasma HDL, LDL and triglycerides; and categorical variables: antihypertensive medication use (yes vs. no) and diabetes status (yes vs. no) in models (Model 2).

The impacts of total fat intake (%kcal), cholesterol-lowering medication use and hormone therapy use were also examined in Model 1 or 2, whereas these factors were not included in the final analyses due to non-significant confounding effects.

All p-values were two-sided. $P<0.05$ was considered statistically significant.

3.4.3 Results

Baseline characteristics of participants

The current study included 3,715 participants, 53.8% of them were women. Men were more likely to developed incident CHD and IS during follow-up than women, i.e. 69.6% of incident CHD events and 58.6% of incident IS events were found in men (**Table 3.4.1**). The participants who developed, compared to those who were free of, incident CHD during follow-up were more likely to be older, ever-smokers, high-educated, diabetic, use anti-hypertensive medication and consumed greater total calories, and had higher BMI, systolic blood pressure and plasma LDL and triglycerides, but lower HDL at baseline. Similar differences were found between participants with and without incident IS. IBRS score and level of individual inflammatory/hemostatic biomarkers were significantly higher among incident CHD and IS cases than non-cases. The proportion of PL fatty acids varied between incident case groups and non-case groups.

Incidence of CHD and IS

A total of 532 participants developed incident CHD during an average of 16.8 years follow-up. The overall crude CHD incidence rate was 8.5 per 1,000 person-years. On the other hand, there were 145 incident IS events during an average of 17.5 years follow-up. The overall crude incident rate of IS was 2.2 per 1,000 person-years.

Assessment of the mediation effects of inflammatory/hemostatic biomarkers for incident CHD

As shown in **Table 3.4.2**, higher IBRS score was significantly associated with increasing risk of incident CHD (P<0.001), adjusting for age, gender, total energy intake, drinking and smoking status, physical activity and education level. PL 18:0 and 20:3ω6 fatty acids were significantly and positively associated with incident CHD, whereas an inverse association was observed for both of PL 17:0 and 20:4ω6 fatty acids in relation to incident CHD (**Table 3.4.3**).

Based on the above findings, the relations of PL 17:0, 18:0, 20:3ω6 and 20:4ω6 fatty acids with IBRS score were further examined (**Table 3.4.4**). Significantly positive associations were found for 18:0 and 20:3ω6 fatty acids in relation to IBRS. However, neither of 17:0 or 20:4ω6 fatty acid was associated with IBRS.

Finally, PL 18:0 and 20:3ω6 fatty acids and IBRS score were simultaneously included in the models as appropriate to predict incident CHD. As shown in **Table 3.4.5**, increasing IBRS significantly mediated the positive association of PL 18:0 and 20:3ω6 fatty acids with incident CHD. Each unit (% of total PL fatty acids) increment of PL 18:0 fatty acid was associated with about 14% (95%CI=6%-23%) reduced risk of incident CHD. At least 20.2% of such effect of 18:0 fatty acid was mediated by per unit increment of IBRS (P<0.001 for the mediation effect). Notably, the statistically significantly positive relation (HR=1.17, 95%CI=1.05-1.31) between PL 20:3ω6 fatty acid and risk of incident CHD was greatly reduced (HR=1.10, 95%CI=0.98-1.23) after adjusting for

IBRS, indicating practically complete mediation effect of IBRS on this positive relation (P<0.001 for the mediation effect).

Similar but weaker mediation effect was found when VIIIc, WBC and fibrinogen were examined individually. Specifically, 8.5%, 11.6% and 9.3% of the positive association between PL 18:0 fatty acid and incident CHD was mediated by each unit increment of levels of VIIIc, WBC and fibrinogen, respectively. However, the practically complete mediation effect of IBRS on the association between PL 20:3ω6 fatty acid and incident CHD was primarily attributed to WBC (data not shown). In addition, both of PL 17:0 and 20:4ω6 fatty acids were inversely related to incident CHD via weak but significant mediation effect of lower WBC (mediation effect size=11.6% and 4.6% for 17:0 and 20:4ω6 fatty acids, respectively; both P<0.001). In contrast, PL 17:0 and 20:4ω6 fatty acids were not associated with levels of VIIIc and fibrinogen and therefore no mediation effect was further examined.

Assessment of the mediation effects of inflammatory/hemostatic biomarkers for incident IS

The mediation effects of IBRS were also assessed for incident IS. However, only about 6.6% of the positive association between PL 16:1ω7 fatty acid and incident IS was mediated by each unit increment of IBRS (**Figure 3.4.1**, P<0.001), which was attributed to the effect of VIIIc and WBC (data not shown). Fibrinogen was positively, but not statistically significantly, related to IS in the current population and therefore no mediation effect of fibrinogen was tested. Similar null results were observed for associations between other PL fatty acid components and incident IS (data not shown).

Sensitivity analyses

In the first part of sensitivity analyses, all of the above associations were re-examined by tracking incident CHD and IS events through year 1999 only. Similar findings were observed for incident CHD, whereas PL fatty acids were not associated with risk of incident IS tracked through year 1999 and therefore no mediation effect of inflammatory/hemostatic biomarkers was tested. Further, the associations of inflammatory/hemostatic biomarkers, or plasma PL fatty acids, with incident CHD/IS were all significantly attenuated by additionally controlling for antihypertensive medication use, BMI, systolic blood pressure, plasma HDL, LDL and triglycerides, and diabetes status (data not shown).

3.4.4 Discussion

The pathophysiology of CHD and IS is generally characterized by atherosclerosis, a chronic disease condition in which the wall of arteries thickens due to the deposition of oxidized fat components (e.g. ox-LDL) and subsequent hemostasis dysfunction and inflammation. In this regard, different fatty acids may contribute to the CHD and IS risk not only as the important compositions of lipid compounds, but also by actively regulating inflammation and hemostatic factors. Based on well-established biological mechanisms[278], the pairwise associations between fatty acids, inflammation/hemostasis, and risk of CHD/IS have been evaluated in a few epidemiological studies, including several ARIC studies[77, 191, 284, 291, 319, 320];[16, 18, 37, 203, 275, 277, 280-282]. However, the development of CHD and IS events is usually a years-long process, during which various confounders can be involved, especially among a free-living population. Although it has

been widely proposed and supported, from a molecular perspective, that inflammation and hemostasis are prominent link between fatty acids and CHD/IS, no population-based study directly addressed the mediation effect (and to how much extent) of inflammation and hemostasis on the association between fatty acids and incident CHD/IS. The current study extended previous evidence and showed that inflammation/hemostasis, reflected by levels of VIIIc, WBC and fibrinogen, mediated the relations between plasma PL fatty acids and incident CHD/IS in a cohort of middle-aged to elderly whites. Specifically, after controlling for age, sex, education level, total energy intake, drinking and smoking status, and physical activity, participants with greater PL fractions of 20:3ω6 fatty acid at baseline had higher risk of developing incident CHD during over 15-year follow-up; and elevated inflammation/hemostasis completely mediated this positive association. Similar but weaker mediation effect of inflammation/hemostasis was also found for PL 18:0 fatty acid in relation to incident CHD, and PL 16:1ω7 fatty acid in relation to incident IS. However, in contrast, baseline PL components of 17:0 and 20:4ω6 fatty acids were inversely associated with risk of incident CHD via lower WBC, but not VIIIc or fibrinogen.

In examining the health effects of fatty acids on CVD risk, it is common to see inconsistent findings. SFA are generally considered to be pro-atherogenic or pro-inflammatory, such as a SFA-rich diet may result in higher serum/plasma LDL, compared to the MUFA or PUFA-rich diets[66-68]. However, not all SFA are created the same and individual SFA could have different health effects. There have been arguments that compared to 16:0 fatty acid, 18:0 fatty acid may be less unhealthy and probably due to

less incorporation into triglyceride and cholesterol ester but more into phospholipids[47]. And in a systematic review of epidemiological studies, higher dietary intake of 18:0 fatty acid has been concluded may be associated with lower LDL level, compared to other SFA[81]. In contrast, however, in the current ARIC population, we observed a positive relation of IBRS and incident CHD with PL composition of 18:0 fatty acid, but not 16:0 fatty acid. This is also supported by some previous studies from both of population and molecular perspectives[81, 326]. For example, high intake of 18:0 fatty acid, when exceeded 9% of total energy intake, may result in increasing level of fibrinogen[81], and *in vitro* treatment of 18:0 fatty acid significantly increased the expression of ICAM-1, a biomarker for inflammation-associated endothelial dysfunction, via NF-κB cell-signaling pathway[326]. Similar discrepancies were also found for other fatty acids in the present study. Both of 17:0 and 20:4ω6 fatty acids were inversely associated with WBC and incident CHD, but not VIIIc and fibrinogen; and 18:2ω6 and ω3 PUFA were inversely associated with IBRS, but not with incident CHD. In line with our findings, a prospective case-control study showed that serum PL 17:0 fatty acid was inversely correlated to serum concentration of plasminogen activator inhibitor-1 (a biomarker linked to thrombosis), but was not associated with increasing risk of myocardial infarction[89]. Although some population-based study supported our results by reporting inverse associations between blood fraction of 20:4ω6 fatty acid and inflammation[122], this is opposed to the widely shown pro-atherogenic characteristics of 20:4ω6 fatty acid due to its pro-inflammatory metabolites--eicosanoids[116]. However, one possible explanation is that not all actions of eicosanoids promote inflammation; Prostaglandin PGE2, an

eicosanoid, may inhibit the production of inflammatory leukotrienes, while induce the production of lipoxin A4 to resolve inflammation[327]. Additionally, consistent with the positive relations we found for PL 20:3ω6 fatty acid with IBRS and incident CHD, Enzenbach et al. observed that higher erythrocyte 20:3ω6 fatty acid was related to higher CRP and lower adiponectin among middle-aged German women and men[328]. And Warensjo et al. also linked higher proportion of serum esterified 20:3ω6 fatty acid to greater cardiovascular mortality[329]. However, since several eicosanoid metabolites of 20:3ω6 fatty acid are anti-inflammatory, the anti-thrombotic potential of 20:3ω6 fatty acid was also proposed from a molecular perspective[125, 330].

Although plasma PL fatty acids have been found to reflect the dietary intakes of corresponding components[9, 83, 84], their cardiovascular health effects may be influenced by, or the influence of, other nutritional factors consumed from mixed foods. Given that other functional nutrients may also modulate inflammation/hemostasis and other CVD pathophysiology, it is not clear to what extent the PL fatty acids/CVD associations can be explained by inflammation/hemostasis. Meanwhile, endogenous fatty acids presented in different tissues (e.g. blood vs. adipose) or as different formats (e.g. free vs. incorporated in to PL) are likely to have diverse associations with CVD risk, which may not necessarily be via regulating inflammatory/hemostatic processes. Some inflammatory and hemostatic factors may be effective markers for CVD risk, but not necessarily in the causal pathway. Especially, inflammation and hemostatic dysfunction were among a variety of CVD risk factors that linked to fatty acid, and they are closely intertwined with other pathologies, such as oxidation. In another words, the fact that one fatty acid is

associated with one pathological biomarker may not be applied to the other biomarker, and also not necessarily lead to the development of incident CVD events due to variations of daily life confounders and population characteristics. Therefore, exploring the mediation effect of inflammation and hemostasis, represented by specific biomarkers may be helpful for understanding the linkage between PL fatty acid component and incident CHD/IS in a free-living population and clarify the study discrepancy. Indeed, among this ARIC population, we found VIIIc, WBC and fibrinogen together explained completely the positive association between PL 20:3ω6 fatty acid and incident CHD, but only about 20% of the positive association between PL 18:0 fatty acid and incident CHD. This suggested that other pathologies besides inflammation/hemostasis may be involved in the effects of PL 18:0 fatty acid. However, on the other hand, since many molecular factors participate in these processes and none of them can be considered to solely represent inflammation/hemostasis, it is plausible to speculate that other biomarkers, in addition to VIIIc, WBC and fibrinogen, are also likely to be remarkable surrogates. Similarly, WBC, but not VIIIc and fibrinogen, was found to mediate 11.6% and 4.6%, respectively, of the inverse relations of PL 17:0 and 20:4ω6 fatty acids with incident CHD in the current study. Blood coagulation pathophysiology reveals that upon the activation by thrombin, VIIIc is released from von Willebrand factor (vWF) and interacts with factor IXa to activate factor X and subsequently more thrombin, which further converts fibrinogen into fibrin. Insofar VIIIc and fibrinogen are known as essential factors in coagulation cascade, while WBC is generally linked to inflammation, our findings implied that anti-inflammation may be more important for the beneficial role of

higher proportion of PL 17:0 and 20:4ω6 fatty acids. However, notably, inflammation and hemostasis, as well as other pathological conditions (e.g. oxidative stress or endothelial dysfunction), are closely intertwined. For example, fibrinogen level is also found to be elevated in response to inflammation as an acute phase reactant[331], while WBC can be raised under a few disease circumstances, etc. Therefore, it appears, to some extent, arbitrary to appoint or classify biomarkers into any specific conditions. Future studies are warranted to elucidate these discrepancies.

Different from incident CHD, we found that incident IS was positively predicted by PL 16:1ω7 fatty acid. Consistent with our results, a case-control study showed that patients with cerebral infarction, a kind of IS, had significantly higher level of 16:1ω7 fatty acid in adipose tissue[332], whereas a recent prospective study in an elderly cohort reported no association between PL 16:1ω7 fatty acid and CHD outcomes[333]. These discrepancies are, on one hand, likely due to the diverse functions of fatty acids. For instance, supplementation of 16:1ω7 fatty acid may result in lower HDL level than supplementation of 16:0 fatty acid[334]. And it is known that, in addition to dietary sources, the PL fraction of 16:1ω7 fatty acid also depend on *de novo* lipogenesis via the activity of Stearoyl-CoA desaturase-1 (SCD-1), which process is known to be confounded by a few biological or lifestyle factors[333]. However, on the other hand, although CHD and IS are generally viewed as diverse manifestations of atherosclerosis, the discrepancies on the relations of various fatty acids with CHD versus IS implied potential differentiations of disease pathophysiology. Taking all these possibilities into account, it is not surprising to see in ARIC population that only 6.6% of the positive relation between PL 16:1ω7 fatty

acid and incident IS was mediated by IBRS, which is much smaller than that found for fatty acids/CHD associations. Therefore, it is reasonable to speculate that inflammation and hemostasis may be less important for the pathology of PL 16:1ω7 fatty acid in relation to IS. This hypothesis has been supported, to some extent, in animal model that in contrast to 18:1ω9 fatty acid, adipocyte-derived 16:1ω7 fatty acid did not induce the inflammation in macrophages and adhesion responses in endothelial cells[335].

Against the strengths of the current report, such as the novelty of our study question, and the large sample size and long-term follow-up of our study population, several limitations should also be pointed out. To begin with, testing for mediation effect was based on priori assumptions of causality in all relation paths. However, the real causality between plasma PL fatty acids, inflammation/hemostasis, and incident CHD/IS may still not be established even with the current prospective study design; especially, both of the plasma PL fatty acid profile and inflammatory/hemostatic biomarkers were measured at baseline. Meanwhile, one time-point measurements of these factors may not well capture the reality. Although we did statistically rule out this possibility by testing whether these variables were time-dependent, among a free-living population, it is still very likely that they may suffer from variations due to biological metabolisms in human body and people's lifestyle changes. The procedure of mediation test, which relies largely on the statistical significance in each relation paths may be another issue, and therefore may conceal some of the true, but weak, associations. Only three inflammatory/hemostatic biomarkers were used in the present analyses, which may not necessarily represent the whole inflammation and hemostasis process, while the specificity of each biomarker for

each pathological condition may also be challenged as described herein. In addition, considering the smaller number of IS events than incident CHD identified during follow-up, the weaker and fewer findings for incident IS may need to be explained with more caution. And since only middle-aged to old whites were included in the current study, our results may not be generalized to populations of other age-groups and ethnicity.

In summary, among the current ARIC population, we demonstrated that inflammation/hemostasis, as reflected by VIIIc, WBC and fibrinogen, completely or partially mediated the associations between several PL fatty acids and incident CHD or IS. The discrepancies of the mediation effects across different fatty acids, inflammatory/hemostatic biomarkers, and CVD outcomes are noteworthy, which supported the complicated molecular-based evidence on the involvement of fatty acids in CVD pathophysiology. Future studies are warranted to confirm out findings by overcoming the above limitations, and further elucidate the underlying mechanisms. However, the current study may also suggest that, in the clinical practice, any specific relations between fatty acids and pathological biomarkers should be used with cautions to imply subsequent development of CVD events. Finally, it is interesting that the associations of inflammation/hemostasis, or plasma PL fatty acids, with incident CHD/IS were significantly attenuated by further adjusting for antihypertensive medication use, BMI, systolic blood pressure, plasma HDL, LDL and triglycerides, and diabetes status. Such findings indicated the potential mediation effects of these risk factors, for which, however, a formal statistical test may be of future interest.

Table 3.4.1 Participants' characteristics at baseline by groups of incident coronary heart disease (CHD) and ischemic stroke (IS) developed during follow-up through year 2007: ARIC (n=3,715)

	No CHD (n=3,183)	CHD (n=532)	No IS (n=3,570)	IS (n=145)
Age (years)	53.5±5.6[a]	55.5±5.4	53.7±5.6	57.6±5.5
Men (%)	43.4	69.6	45.7	58.6
Education level>high school (%)	40.4	42.9	40.7	42.1
Ever-drinker (%)	95.7	95.9	95.8	93.8
Ever-smoker (%)	60.5	71.6	62.0	64.1
Physical active (%, sport index≥2)	77.8	79.1	78.0	77.2
Total energy intake (kcal)	1626±577	1729±619	1639±582	1681±631
BMI (kg/m^2)	26.8±4.6	28.1±4.3	27.0±4.6	27.6±4.8
Systolic blood pressure (mmHg)	117.9±15.9	123.8±17.2	118.4±16.1	125.4±18.0
Plasma HDL-C (mmol/L)	1.4±0.5	1.1±0.4	1.4±0.4	1.2±0.4
Plasma LDL-C (mmol/L)	3.5±1.0	3.9±1.0	3.5±1.0	3.8±1.0
Plasma triglycerides (mmol/L)	1.4±0.9	1.8±1.3	1.4±1.0	1.9±1.6
Anti-hypertensive medication use (%)	19.6	28.6	20.4	34.5
Diabetes (%)	5.4	17.0	6.6	17.9
Phospholipids fatty acids (%total phospholipids fatty acids)				
15:0	0.171±0.041	0.167±0.039	0.171±0.041	0.164±0.043
16:0	25.4±1.7	25.5±1.5	25.4±1.6	25.7±1.7
17:0	8.9±2.8	8.8±3.0	8.9±2.8	8.5±2.5
18:0	13.2±1.2	13.5±1.2	13.3±1.2	13.4±1.3
16:1, ω7	0.64±0.18	0.63±0.18	0.64±0.18	0.68±0.20
18:1, ω9	8.6±1.2	8.6±1.2	8.6±1.2	8.8±1.3
18:2, ω6	22.0±2.7	21.9±2.7	22.0±2.7	21.8±3.0
18:3, ω3	0.15±0.05	0.14±0.05	0.15±0.05	0.14±0.05
18:3, ω6	0.11±0.06	0.11±0.06	0.11±0.06	0.12±0.06
20:3, ω6	3.3±0.8	3.4±0.8	3.3±0.8	3.4±0.7

20:4, ω6	11.5±2.0	11.3±1.9	11.5±1.9	11.4±2.1
20:5, ω3	0.56±0.30	0.58±0.43	0.57±0.33	0.58±0.26
22:6, ω3	2.8±0.9	2.8±0.9	2.8±0.9	2.7±0.9
LC-ω3	3.4±1.0	3.4±1.1	3.4±1.1	3.3±1.0
Inflammatory and hemostatic biomarkers				
IBRS	-0.02±0.62	0.14±0.61	-0.01±0.61	0.24±0.70
VIIIc (%)	121.8±33.3	127.5±35.8	122.2±33.3	132.8±40.5
WBC (×1,000 cells/mm^2)	6.1±1.8	6.6±1.9	6.2±1.8	6.8±2.1
Fibrinogen (mg/L)	290.7±59.6	304.6±63.4	292.1±59.9	307.0±68.7

[a]Mean±SD for all such values

Table 3.4.2 Hazard ratio (95% CI) of incident CHD in relation to IBRS: ARIC [a] (n=3,715)

IBRS quintiles								
Q1[b]	Q2	Q3	Q4	Q5	β_{trend}[c]	P_{trend}[c]	β_{con}[d]	P_{con}[d]
1	1.11 (0.81, 1.53)	1.23 (0.90, 1.67)	1.57 (1.16, 2.11)	1.99 (1.48, 2.69)	0.432	<0.001	0.403	<0.001

[a] Model was adjusted for age, gender, total energy intake, drinking status, smoking status, physical activity and education level.
[b] Q1=quintile 1; only quintile 1, 3 and 5 were presented here.
[c] β_{trend} and P_{trend} are weighted average slope and p-value for the linear trend of incident CHD risk across IBRS quintiles.
[d] β_{con} and P_{con} are regression coefficient and p-value of IBRS when entered into models as a continuous variable.

Table 3.4.3 Hazard ratio (95% CI) of incident CHD in relation to PL fatty acids: ARIC [a] (n=3,715)

PL fatty acids	Q1 [b]	Q2	Q3	Q4	Q5	β_{trend} [c]	P_{trend} [c]	β_{con} [d]	P_{con} [d]
15:0	1	1.25 (0.95, 1.64)	1.04 (0.79, 1.37)	1.28 (0.99, 1.66)	0.99 (0.73, 1.33)	0.371	0.78	0.093	0.93
16:0	1	1.36 (1.02, 1.81)	1.27 (0.95, 1.70)	1.32 (0.99, 1.75)	1.12 (0.83, 1.51)	0.011	0.72	0.000	0.99
17:0 [e]	**1**	0.87 (0.67, 1.13)	0.78 (0.59, 1.02)	0.72 (0.55, 0.95)	0.70 (0.53, 0.93)	**-0.061**	**0.008**	**-0.395**	**0.02**
18:0	**1**	1.37 (1.01, 1.86)	1.28 (0.95, 1.74)	1.45 (1.07, 1.95)	1.75 (1.31, 2.35)	**0.164**	**<0.001**	**0.129**	**<0.001**
16:1, ω7 [e]	1	0.92 (0.71, 1.19)	1.02 (0.78, 1.34)	0.90 (0.68, 1.18)	0.93 (0.70, 1.23)	-0.178	0.61	0.022	0.90
18:1, ω9	1	0.90 (0.68, 1.19)	1.02 (0.78, 1.33)	0.81 (0.61, 1.08)	0.94 (0.71, 1.23)	-0.029	0.54	-0.024	0.54
18:2, ω6	1	1.02 (0.78, 1.33)	0.96 (0.73, 1.27)	0.98 (0.74, 1.29)	1.05 (0.80, 1.38)	0.004	0.84	-0.005	0.77
18:3, ω3	1	1.07 (0.82, 1.38)	1.02 (0.79, 1.31)	1.09 (0.84, 1.43)	0.92 (0.68, 1.25)	-0.353	0.76	0.204	0.83
18:3, ω6	1	0.94 (0.71, 1.24)	1.08 (0.80, 1.47)	1.02 (0.76, 1.37)	1.02 (0.76, 1.37)	-0.234	0.80	-0.036	0.97
20:3, ω6	**1**	1.02 (0.75, 1.38)	1.13 (0.85, 1.51)	1.43 (1.08, 1.88)	1.38 (1.04, 1.83)	**0.196**	**0.003**	**0.157**	**0.006**
20:4, ω6	**1**	0.80 (0.61, 1.04)	0.87 (0.67, 1.13)	0.77 (0.59, 1.01)	0.72 (0.55, 0.95)	**-0.059**	**0.03**	**-0.065**	**0.005**
20:5, ω3 [e]	1	1.08 (0.82, 1.41)	1.08 (0.82, 1.44)	1.08 (0.82, 1.42)	1.02 (0.77, 1.36)	0.012	0.96	0.038	0.71
22:6, ω3	1	1.07 (0.82, 1.40)	1.09 (0.83, 1.44)	0.90 (0.67, 1.20)	1.21 (0.92, 1.59)	0.058	0.36	0.036	0.47

[a] Model was adjusted for age, gender, total energy intake, drinking status, smoking status, physical activity and education level.
[b] Q1=quintile 1; only quintile 1, 3 and 5 were presented here.
[c] β_{trend} and P_{trend} are weighted average slope and p-value for the linear trend of incident CHD risk across quintiles of fatty acids.
[d] β_{con} and P_{con} are regression coefficient and p-value of fatty acids when entered into models as a continuous variable.
[e] 17:0, 161ω7 and 20:5ω3 fatty acids were in log-scale when entered into models as continuous variables.

Table 3.4.4 Mean (±SE) IBRS score by quintiles of PL fatty acids: ARIC [a] (n=3,715)

PL fatty acids	Q1 [b]	Q2	Q3	Q4	Q5	β_{trend} [c]	P_{trend} [c]	β_{con} [d]	P_{con} [d]
17:0 [e]	0.05±0.02	0.007±0.02	-0.02±0.02	-0.05±0.02	0.01±0.02	-0.008	0.14	-0.030	0.46
18:0	**-0.12±0.02**	**-0.03±0.02**	**0.01±0.02**	**0.03±0.02**	**0.10±0.02**	**0.070**	**<0.001**	**0.057**	**<0.001**
20:3, ω6	**-0.25±0.02**	**-0.10±0.02**	**0.03±0.02**	**0.12±0.02**	**0.20±0.02**	**0.231**	**<0.001**	**0.201**	**<0.001**
20:4, ω6	-0.00±0.02	-0.01±0.02	0.04±0.02	-0.01±0.02	-0.03±0.02	-0.006	0.32	-0.006	0.25

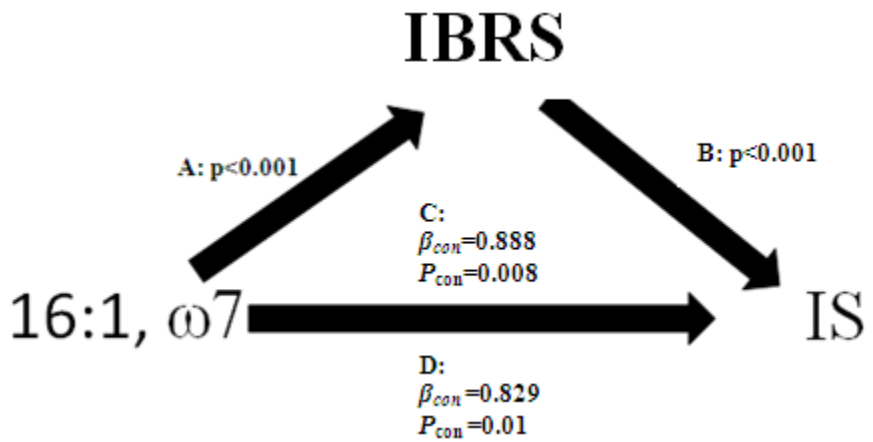
[a] Model was adjusted for age, gender, total energy intake, drinking status, smoking status, physical activity and education level.
[b] Q1=quintile 1; only quintile 1, 3 and 5 were presented here.
[c] β_{trend} and P_{trend} are weighted average slope and p-value for the linear trend of incident CHD risk across quintiles of fatty acids.
[d] β_{con} and P_{con} are regression coefficient and p-value of fatty acids when entered into models as a continuous variable.
[e] 17:0 fatty acid was in log-scale when entered into models as continuous variables.

Table 3.4.5 Hazard ratio (95% CI) of incident CHD in relation to PL fatty acids mediated by IBRS: ARIC [a] (n=3,715)

PL fatty acids *Biomarkers*	From **Table 3.4.2 (**without IBRS)						Mediation effect size[c]	*P*[d]
	β_{con} [b]	HR (95%CI)[e]	P_{con} [b]	β_{con} [b]	HR (95%CI) [e]	P_{con} [b]		
18:0	0.103	1.11 (1.03, 1.19)	0.007	0.129	1.14 (1.06, 1.23)	<0.001	20.2%	<0.001
20:3, ω6	0.091	1.10 (0.98, 1.23)	0.12	0.157	1.17 (1.05, 1.31)	0.006	42.0%[f]	<0.001

[a] Models were adjusted for IBRS, age, gender, total energy intake, drinking status, smoking status, physical activity and education level.
[b] β_{con} and P_{con} are regression coefficient and p-value of fatty acids when fatty acids entered into models as continuous variables.
[c] Mediation effect size: percent of fatty acid-CHD relation mediated by each unit increment of IBRS (mediation size=($\beta_{con\ from\ Table\ 3.4.2}$-$\beta_{con}$)/ $\beta_{con\ from\ Table\ 3.4.2}$);
[d] P-value for the mediation effect.
[e] HR represents the HR of incident CHD for per unit (% of total PL fatty acids) increment of these fatty acids.
[f] Complete mediation effect of IBRS by statistical test procedure.

Figure 3.4.1 Test of mediation effect of IBRS on the association between PL 16:1ω7 fatty acids and IS: ARIC[a].



Path A: Test of whether fatty acid is the predictor of IBRS (generalized linear regression).
Path B: Test of whether IBRS predict IS (Cox proportional regression).
Path C: Test of whether fatty acid predicts IS (Cox proportional regression without IBRS).
Path D: Test of whether fatty acid predicts IS via the mediation of IBRS (Cox proportional regression with IBRS).

[a] 16:1ω7 fatty acid was entered into models in log-scale.
Models were adjusted for age, gender, total energy intake, drinking status, smoking status, physical activity, education level and IBRS as appropriate.

CHAPTER 4. SUMMARY

The primary aim of this research was to explore and better understand the comprehensive associations between fatty acids and inflammation across the lifecycle. The rational was based on the broadly reported, but still inconclusive, evidence that linked fatty acids (either dietary or endogenous) to the progression of CVD risk, including intertwined pathologies such as inflammation, hemostatic dysfunction and oxidative stress[39-42, 48-50, 58]. Especially, the diverse cardiovascular health effects of individual fatty acids have not been well studied among populations of different ages. Meanwhile, the research motivation for these studies also came from the increasing childhood obesity prevalence[336], as well as the promising role of gene-diet interaction on CVD risk. Based on the availability of data, a total of 13 individual fatty acids were studied among three populations (**Table 4.1**). This chapter summarized the main findings of each manuscript, and then further discussed the public health implications of these findings by comparing between manuscripts and to the previous evidence.

Table 4.1 List of fatty acids studied in this dissertation

Fatty acid measure-ments	Populations			
	Adolescents	CARDIA adults	ARIC adults	ARIC adults
	PL	Diet	Diet	PL
15:0	√	×	×	√
17:0	√	√	×	√
16:0	√	√	√	√
18:0	√	√	√	√
16:1ω7	√	√	√	√
18:1ω9	√	√	√	√
18:2ω6	√	√	√	√
18:3ω3	√	√	√, Not	√

18:3ω6	×	×	differentiated	√
20:3ω6	√	×	×	√
20:4ω6	√	√	√	√
20:5ω3	√	√	√	√
22:6ω3	√	√	√	√

√: studied; ×: not studied.

4.1 Summary of Manuscript 1

This cross-sectional study, conducted among adolescents with an average age of 15-year (range=12-18 years), was to examine whether and how overweight status modifies the relations between serum markers of dairy fats (i.e. 15:0 and 17:0 fatty acids) and inflammation/oxidative stress. As expected, among overweight adolescents, but not their normal weight counterparts, inverse associations were found between dairy fatty acids and three biomarkers of inflammatory and oxidative stress, i.e. CRP, F2-iso and 15-keto. In contrast, independent of weight status, dairy fatty acids were inversely related to the inflammatory biomarker IL-6 in all participants. Notably, these anti-inflammatory characteristics of dairy fatty acids were not influenced by other nutrients that may contribute to the health role of dairy products, e.g. calcium, vitamin D, protein, total flavonoids, and ω3 fatty acids. Similar study question and statistical methods were applied to an additional analysis of other serum fatty acids, in which significant effect modification of weight status was also observed. The main findings in the overweight group, but absent in normal weight group, included the positive relations of 18:0 fatty acid with CRP, F2-iso and 15-keto; the positive relation of 20:3ω6 fatty acid with IL-6; and the inverse relations of 20:4ω6 and 22:6ω3 fatty acids with IL-6.

4.2 Summary of Manuscript 2

Manuscript 2 primarily explored the effect of a genetic variant, i.e. Pro12Ala polymorphism, on the cross-sectional relations between dietary PUFA intake and three inflammatory biomarkers (i.e. IL-6, CRP and fibrinogen). The study population included biracial middle-aged adults (age range=37-66 years, mean age 45 years) participating in the year 20 exam of the CARDIA study. Interestingly, it was found that dietary intakes of 20:4ω6, 20:5ω3 and 22:6ω3 fatty acids were associated with IL-6 level differently by both of gender and Pro12Ala genotype. In women, higher dietary intakes of these fatty acids were related to lower IL-6 level among Ala allele carriers. In contrast, these PUFA/IL-6 relations were positive among male Ala carriers, and absent among female Pro homozygotes. Male Pro/Pro homozygotes who consumed more 20:5ω3 and 22:6ω3 fatty acids tended to have a lower IL-6 level. Additionally, independent of the effect of Pro12Ala polymorphism, dietary intake of α-linolenic acid (ALA, 18:3ω3) was inversely related to level of IL-6 in women, but not in men. Pro12Ala genotype did not modify any dietary PUFA intake in relation to CRP or fibrinogen, whereas higher intake of 20:4ω6 fatty acid was positively linked to CRP and fibrinogen in both genders.

4.3 Summary of Manuscript 3

In the prospective study described in Manuscript 3, a cohort of 15,792 middle-aged adults (baseline age range=44-66 years, mean age 54 years) who enrolled in the ARIC study were tracked for over 15 years. The objective of this study was to examine the interactions between dietary fatty acid intake and inflammatory/hemostatic factors in

relation to incident CHD and IS. Among the 13,734 participants included in the current analyses, 2,148 incident CHD events and 679 incident IS cases were identified during follow-up. Dietary intakes of 18:2ω6 and 20:4ω6 fatty acids were found to modify the associations between serum albumin and incident CHD/IS. The prediction of low serum albumin level on incident CHD/IS was attenuated with increasing intake of 18:2ω6 fatty acid or decreasing intake of 20:4ω6 fatty acid. However, the associations between other examined hemostatic/inflammatory biomarkers (i.e. vWF, VIIIc, fibrinogen and WBC) and incident CHD/IS were not modulated by any dietary fatty acids.

4.4 Summary of Manuscript 4

Although it has been widely proposed that fatty acids may influence CVD risk via promoting or preventing inflammation and hemostatic dysfunction, there has not been a population-based study so far that has addressed this hypothesis. The study in Manuscript 4 used the data from a sub-group of 3,715 ARIC participants enrolled at the Minnesota field center and had plasma PL fatty acid measurements, to examine whether and to how much extent, inflammation/hemostasis mediated the relation of fatty acids with incident CHD and IS. During 15-years of follow-up, 532 and 145 participants developed incident CHD and IS, respectively. In concordance with an *a priori* hypothesis, inflammation and hemostasis, represented by levels of VIIIc, WBC and fibrinogen, mediated the positive associations of PL 18:0 and 20:3ω6 fatty acids with incident CHD. A similar but less significant pattern was found for PL 16:1ω7 in relation to incident IS. Lower WBC count,

but not VIIIc or fibrinogen, partially explained the inverse relations of PL 17:0 and 20:4ω6 fatty acids with incident CHD.

4.5 Overall discussion

Several physical and physiological changes occur in the human body over time, including body composition, metabolism, immune function and other system changes. For example, elderly may be more susceptible to diseases compared to young populations, whereas adolescents, compared to older adults, may not have a lifetime of exposure to adverse risk factors, including smoking, high calorie and/or saturated fat intake, and physical inactivity. These differences across the lifecycle may further influence the diverse relations between individual fatty acids, inflammation and the development of CVD. In the current studies, several common (e.g. age, race, physical activity, total energy intake, etc) or different (e.g. Tanner score, smoking, alcohol intake, medication use, etc.) confounding factors have been accounted for in statistical models; however, residual confounding may not be ruled out. In addition to the previous discussion for each study, further discussion of the study implications are noteworthy.

4.5.1 Dietary vs. blood phospholipids fatty acids

Fatty acids have been well known not only contribute to energy storage and cellular structure build-up, but also participate in cell signaling transduction and gene regulation. In examining their health effects, dietary and blood PL fatty acid components are often used, due to relatively easy and convenient measurements. PL fatty acids have been found to mirror the dietary intakes of corresponding contents for at least short-term[9, 83, 84],

especially for those that cannot be synthesized in human body (e.g. 15:0 and 17:0 fatty acids and PUFA) compared to those that can be generated endogenously (e.g. 18:0 fatty acid). In this regard, it is not surprising that dietary and PL fatty acids may share a few common health effects. However, dietary fatty acids may also directly exert their functions without being metabolized, while PL fatty acids, as being incorporated into cell membrane, may act in a slower manner than free fatty acids[337]. For example, dietary free fatty acids may influence the cardio function via interfering ionic channels in addition to regulating gene expressions, while PL fatty acids contribute largely to the modulation of membrane fluidity, hormones and genetic factors[337]. Meanwhile, dietary and PL fatty acids may regulate gene expressions via different signaling pathways, or they may be differently influenced by other endogenous and environmental factors, which will be discussed later. Therefore, differential health effects of dietary versus PL fatty acid components can be expected. Indeed, the current dissertation revealed such discrepancies.

PL 17:0 fatty acid, as a biomarker for dairy fat consumption, was studied among both of ARIC adult Minnesotans and adolescents. Consistent in these two studies, higher proportion of PL 17:0 fatty acid was associated with lower inflammation, oxidative stress, hemostatic dysfunction, or the subsequent CVD risk. Our findings support previously reported findings on the potential cardioprotection of PL 17:0 fatty acid[89, 211, 217]. However, no such protection was found for dietary 17:0 fatty acid as studied among CARDIA adults in relation to inflammation. Similar differences were also observed for 20:4ω6 fatty acid. Specifically, PL 20:4ω6 fatty acid was inversely associated with

inflammatory biomarker IL-6 level among overweight adolescents, and inversely related to WBC count and incident CHD among ARIC adult Minnesotans. In contrast, higher consumption of 20:4ω6 fatty acid enhanced the prediction of low serum albumin level on CVD incidence in ARIC adults, and was generally pro-inflammatory in CARDIA adults (except in female Pro12Ala polymorphism Ala allele carriers). Interestingly, as described in the manuscripts, both pro- and anti-inflammatory roles of 20:4ω6 fatty acid were reported previously in population- or molecular-based studies[116, 122, 327].

4.5.2 Diversity of individual fatty acids

It has been very clear that individual fatty acids are not created the same. The findings in this dissertation, again, revealed such diversity, in addition to the widely shown differences between different fatty acid families, i.e. SFA, MUFA and PUFA.

Dairy fatty acids (i.e. 15:0 and 17:0) versus other SFA can be a typical example in this regard. Among the overweight adolescents, we found that PL dairy fatty acids were inversely, while PL 18:0 fatty acid was positively related to CRP, F2-iso and 15-keto inflammatory biomarkers. And in ARIC adults, similar opposite associations with inflammation/hemostasis and incident CHD were found between PL 17:0 and 18:0 fatty acids. Meanwhile, nothing was observed across all four studies regarding the health effect of 16:0 fatty acid; except that in CARDIA adults, dietary 16:0 fatty acid intake was very weakly and positively correlated to CRP level (while adjustment of BMI further attenuated this correlation). It is noteworthy, however, that both of 16:0 and 18:0 fatty acids are actually two of primary fatty acids in dairy products. According to a study

examining Swedish dairy milk in year 2001, 16:0 and 18:0 fatty acids counted for 30.6% and 12.2% of total fatty acids, respectively, while 15:0 and 17:0 fatty acids together only weighed for 1.3% of total fatty acids. Nevertheless, the feature of 15:0 and 17:0 fatty acids is that they cannot be synthesized intrinsically by human body and therefore the PL profile of them relatively well mirrors the dairy consumption[82, 83]. In contrast, 16:0 and 18:0 can both be generated endogenously, while *de novo* lipogenesis may be dependent on many factors, such as body adiposity, dietary consumption of other nutrients (e.g. carbohydrate), etc. Although 18:0 fatty acid was proposed may be less pro-atherogenic than 16:0 fatty acid[47, 81], it was not supported by our findings and some previous evidence[326]. In addition, from the studies in this dissertation, we also observed that compared to 15:0 fatty acid, the health effects of 17:0 fatty acid may be more pronounced in terms of anti-inflammation and cardioprotection. Such trivial differences were also found in the current dissertation between 20:5ω3 and 22:6ω3 fatty acids that the later one may have a greater anti-atherogenic potential than the former one. Greater accumulations of 22:6ω3 than 20:5ω3 in the body[337] may be one explanation. However, future studies are warranted to further elucidate these discrepancies.

4.5.3 Adiposity, genetic variations, gender and age

Obesity (and overweight) most likely results from positive energy balance, thus leading to excessive body fat accumulation, which hampers adipocyte differentiation and promotes adipocyte hypertrophy and dysfunction[206]. As a consequence, macrophages infiltrate into adipose tissue, secrete various pro-inflammatory cytokines, while inhibit

the production of anti-inflammatory adipokines[206]. On the other hand, free fatty acids, over-produced by hypertrophic adipocytes or induced from dietary overconsumption may activate pro-inflammatory signaling pathways, e.g. Toll-like receptor-4 and NF-κB pathways, which then further amplify the inflammatory process[206]. Studies have linked obesity to altered fatty acid metabolism, inflammation progression and CVD development, and a few metabolic disorders are obesity-associated, such as hypertension, dyslipidemia, insulin resistance, etc.[139, 206, 338]. Higher activity of adipocyte-involved cross-talks and feedback loops in obese individuals may shed light on why we saw enhanced associations of several PL fatty acids with inflammation in overweight adolescents. However, adjustment of BMI did not materially change the dairy fats/IL-6 relation among all studied adolescents, indicating a non-significant confounding effect of BMI. Meanwhile, it is noteworthy that, among ARIC adults, adjustment of BMI and some metabolic measurements (i.e. systolic blood pressure, cholesterol levels, etc.) enhanced the modification effects of dietary 18:2ω6 and 20:4ω6 fatty acids on serum albumin/CHD relations, while others attenuated the mediation effects of inflammation/hemostasis on PL fatty acids/CHD (or IS) associations; and no factors materially changed the Pro12Ala-dietary PUFA interactions in relation to IL-6 level among CARDIA adults. The many faces of adiposity and its related metabolic conditions posing in fatty acids-inflammation-CVD associations deserves further investigations.

In this context, adiposity may interplay with genetic variations, such as Pro12Ala polymorphism. It has been known that carrying Ala allele was significantly associated

with improved endogenous lipid profile, insulin sensitivity and lower risk of type II diabetes, but may be more likely to have higher BMI due to greater potential of accumulating body fat[254, 256, 257]. Therefore, it is plausible to hypothesize that the role of adiposity and its related metabolic conditions can be partially explained by the effect of Pro12ALA genotype, which may be one explanation for the non-significant influence of adjustment of BMI on the Pro12Ala-dietary PUFA interactions in relation to IL-6 level among CARDIA adults. Meanwhile, as opposed to our hypothesis, dietary PUFA intake was found to be related to IL-6 level in Ala allele carriers than Pro homozygotes. Such an inconsistency may be partially explained, however, if the association between Ala allele and greater adiposity truly exists (recall the findings of enhanced associations of several PL fatty acids with inflammation in overweight adolescents).

Another unexpected finding in the current CARDIA study was that none of the dietary SFA and MUFA, as well as 18:2ω6 and 18:3ω3 fatty acids, interacted with Pro12Ala polymorphism in relation to inflammation. One explanation may be the characteristic of PPARγ ligands. However, it remains unclear whether and how these ligands share any identity[339]. Actually, there has been evidence showing that modified fatty acids, such as oxidized fatty acids, may have higher potential in activating PPARγ than native fatty acids[339]. Whether the differences we reported are partially due to the oxidation caused by long-chain PUFAs compared to other fatty acids deserves further study. Further, the non-significant SFA/MUFA-Pro12Ala interactions should also be mentioned. For example, Memisoglu et al. reported that BMI was negatively associated with MUFA intake among

Ala carriers but not Pro/Pro[340], while regardless of Pro12Ala genotype, SFA intake was positively related to BMI among all participants[340]. In contrast, Robitaille et al. found that SFA intake was negatively correlated to HDL and positively correlated to BMI, waist circumference, fasting glucose, etc. among Pro/Pro, whereas all of these associations were absent among Ala carriers[341]. Related evidence is still limited so far. Therefore, future studies are warranted to better clarify the role of Pro12Ala polymorphism.

In addition to genetic variation, gender is also an essential intrinsic player in the fatty acids-inflammation-CVD relation. Women are generally, but not always, at lower risk of CVD than men[342, 343] and results from many studies have revealed that men and women are different in fatty acid metabolism and gene/hormone regulation[249-251, 270]. Indeed, we found gender-specific Pro12Ala-fatty acid interactions in relation to inflammation among CARDIA adults, although we failed to observe such a gender-effect in adolescents and ARIC adults. A large proportion of the gender-difference may be explained by the higher percentage of body fat in women[250] and the different body fat distribution (i.e. "greater accumulation of healthy subcutaneous fat in women than men") between genders[206]. Similar contribution of adiposity may also help explain some age-related health effects. For instance, the age-related CRP level was no longer observed in a study comparing nonobese middle-aged men to young nonobese men with the same amount of visceral adipose tissue[344]. Therefore, it may be the age-associated increasing visceral adipose tissue that resulted in higher CRP level[206], which, again, implies the important role of adiposity. However, we cannot ignore the impact of age. Although we were able to

statistically control for age and some of age-associated factors, there are still many other factors that are beyond the scope of the current dissertation. These factors may include numerous aging-associated changes in cardiac gene expression, cardiovascular physiology, immunity, lifestyle, and social status, for example[345, 346].

4.6 Overall conclusions

The findings from these studies included in this dissertation have enhanced the understanding of the linkage between diverse fatty acids, inflammation and CVD risk among populations in different life stages. The important health beneficial effect of long-chain ω3 fatty acids (especially 22:6ω3) was further confirmed, while new evidence was uncovered, such as the cardioprotective potentials of dairy fatty acids (i.e. 15:0 and 17:0), the proinflammatory nature of 16:1ω7 and 20:3ω6 fatty acids, and the double-faces (i.e. pro- and anti-inflammatory) of 20:4ω6 fatty acid, etc. Dietary fatty acids are not consumed individually and the health effects of fatty acids may depend on the total matrix of a food as well as the whole dietary pattern. However, by studying individual fatty acids and their interplay with other factors, results from the studies reported in this dissertation have shed new light on several underlying mechanisms for fatty acids in relation to CVD-associated pathological conditions (i.e. inflammation, oxidative stress, hemostatic dysfunction) and CVD endpoints. Such findings may be helpful in providing evidence-based dietary recommendations or advising food manufacturers (e.g. substitute one fatty acid with another in food products). Finally, the crucial role of inflammation in CVD development across the lifecycle was highlighted in this dissertation. The

importance and a promising effect of controlling inflammation progression for CVD prevention were therefore suggested.

Future studies are warranted and should be conducted to overcome potential study limitations (e.g. study design, population sample size, etc.), to confirm the current findings, clarify the inconsistencies, and further elucidate the fatty acid-inflammation-CVD associations.

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