International Journal of Vascular Medicine

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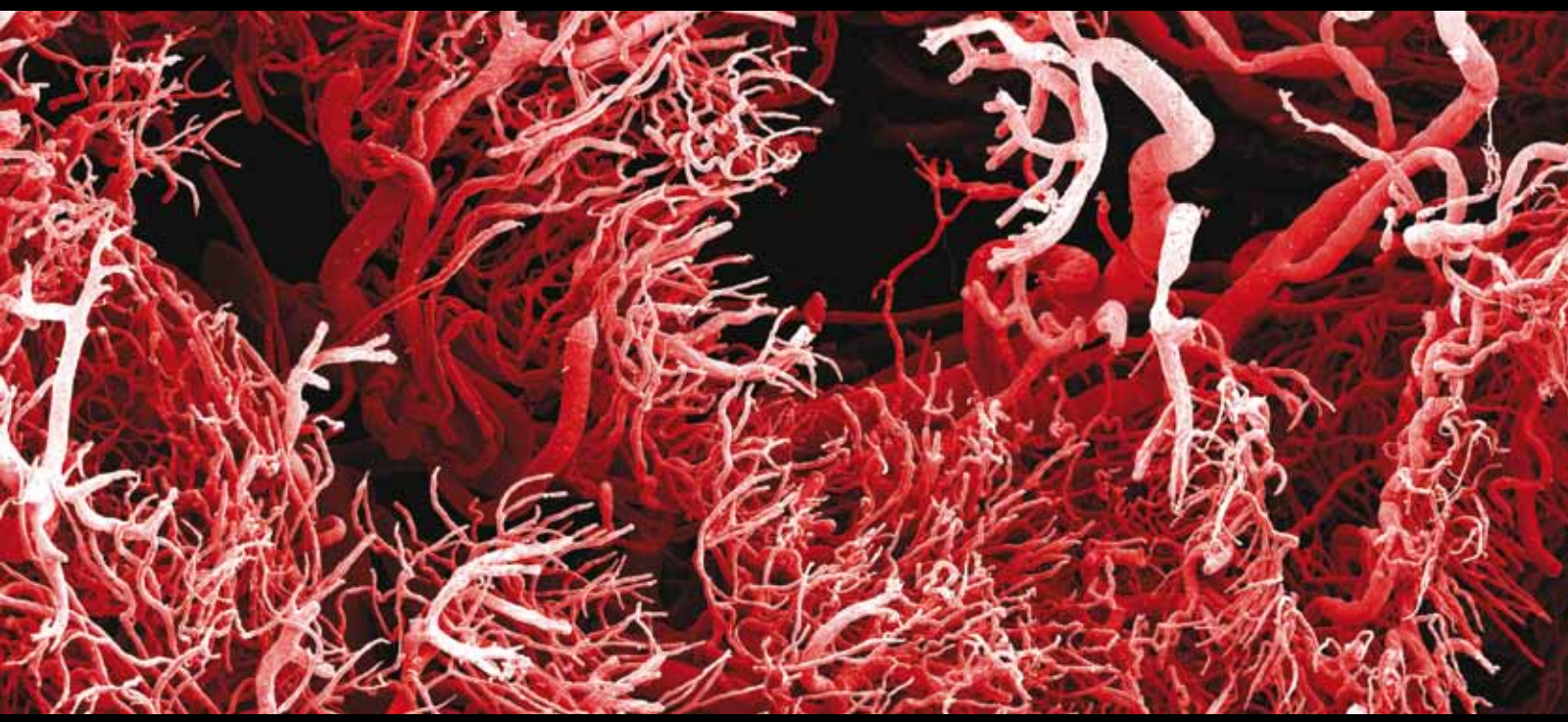
Guest Editors: M. Castro Cabezas, Kathleen M. Botham, John C. L. Mamo, and Spencer D. Proctor



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Editorial Board

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Editorial

Novel Aspects of Nonfasting Lipemia in relation to Vascular Biology

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Several cardiovascular risk factors have been described during the last decades. Postprandial hyperlipidemia has been recognized as an important factor contributing to atherogenesis; however chylomicron remnants have not been used widely in risk equations. One of the probable causes for this lack of consideration in clinical practice may be that the metabolism of chylomicrons and their remnants has been rather difficult to study and, more so, to modulate in vivo. The current issue of the International Journal of Vascular Medicine provides novel insight into the metabolic changes of chylomicrons and their remnants in relation to vascular damage. The paper by G. H. Tomkin and D. Owens "*The chylomicron: relationship to atherosclerosis*" provides an excellent overview of the molecular mechanisms involved in chylomicron synthesis and metabolism in humans. The paper by B. Klop et al. "*Understanding postprandial inflammation and its relationship to lifestyle behaviour and metabolic diseases*" describes novel aspects related to inflammation in different clinical conditions and under variable lifestyle situations. Following this line of inflammatory-dependent aspects of chylomicrons, the paper by M. Cao et al. "*Dual AAV/IL-10 plus STAT3 anti-inflammatory gene delivery lowers atherosclerosis in LDLR KO mice, but without increased benefit*" further explores aspects of cholesterol metabolism involving different molecular targets important for both hepatic and intestinal cholesterol metabolism. S. A. Lopez et al. proceed with a paper investigating the effects of oxidized chylomicron remnants on monocyte activation and the effects of the antioxidant drug probucol on this interaction "*The oxidative state of chylomicron remnants influences their modulation of human monocyte activation.*" L. D. Roche and et al. provide evidence suggesting that postprandial hypertriglyceridemia simulated by an infusion with artificial TG-rich lipoproteins (Lipofundin) induces oxidative stress and atherosclerotic lesions in an animal model prone to develop atherosclerosis "*Lipofundin-induced hyperlipidemia promotes oxidative stress and atherosclerotic lesions in New Zealand white rabbits.*"

The paper by M. Napolitano et al. "*Phospholipase A2 mediates apolipoprotein-independent uptake of chylomicron remnant-like particles by human macrophages*" includes novel work showing that macrophage lipolytic enzymes mediate the internalization of postprandial TG-rich lipoproteins and that secretory phospholipase A2 ($sPLA_2$) and cytosolic PLA2 play a more important role than extracellular lipoprotein lipase-mediated TG hydrolysis. M. M. Pallebage-Gamarallage et al. describe in the following investigation "*A diet enriched in docosahexanoic acid exacerbates brain parenchymal extravasation of Apo B lipoproteins induced by chronic ingestion of saturated fats,*" how dietary interventions can lead to modulation of cerebrovascular inflammation by oxidative stress. A. Van der Wiel provides a comprehensive

update on the effects of alcohol ingestion on fasting and postprandial triglyceridemia "*The effect of alcohol on postprandial and fasting triglycerides*", which is followed by a paper by B. Klop et al. "*AT1 receptor gene polymorphisms in relation to postprandial lipemia*" showing a novel relationship between polymorphisms of genes involved in the regulation of blood pressure with postprandial lipemia. J. P. H. van Wijk and M. Castro Cabezas contributed with an update on the effects of antiretroviral therapy in HIV-infected patients on TG metabolism "*Hypertriglyceridemia, metabolic syndrome, and cardiovascular disease in HIV-infected patients: effects of antiretroviral therapy and adipose tissue distribution.*" Finally, G. Charach et al. provided a review describing the effects of bile acid secretion and intestinal absorption on plasma lipoproteins and coronary atherosclerosis.

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Research Article

A Diet Enriched in Docosahexanoic Acid Exacerbates Brain Parenchymal Extravasation of Apo B Lipoproteins Induced by Chronic Ingestion of Saturated Fats

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Chronic ingestion of saturated fatty acids (SFAs) was previously shown to compromise blood-brain barrier integrity, leading to brain parenchymal extravasation of apolipoprotein B (apo B) lipoproteins enriched in amyloid beta. In contrast, diets enriched in mono- or polyunsaturated (PUFA) oils had no detrimental effect. Rather, n3 and n6 oils generally confer protection via suppression of inflammation. This study investigated in wild-type mice if a PUFA diet enriched in docosahexanoic acid (DHA) restored blood-brain barrier integrity and attenuated parenchymal apo B abundance induced by chronic ingestion of SFA. Cerebrovascular leakage of apo B was quantitated utilising immunofluorescent staining. The plasma concentration of brain-derived S100β was measured as a marker of cerebrovascular inflammation. In mice fed SFA for 3 months, provision thereafter of a DHA-enriched diet exacerbated parenchymal apo B retention, concomitant with a significant increase in plasma cholesterol. In contrast, provision of a low-fat diet following chronic SFA feeding had no effect on SFA-induced parenchymal apo B. The findings suggest that in a heightened state of cerebrovascular inflammation, the provision of unsaturated fatty acids may be detrimental, possibly as a consequence of a greater susceptibility for oxidation.

1. Introduction

Accumulating evidence supports the hypothesis that dietary behaviour and in particular ingestion of fats contribute to Alzheimer's disease (AD) onset and progression. The work in [1] reported that the prevalence of sporadic and late-onset AD in >65 years of age subjects correlated with fat intake and was higher in Western countries, compared to Africa or Asia. Population and clinical studies also suggest that regular consumption of saturated fatty acids (SFAs) and trans-fatty acids as well as cholesterol is synergistically and positively associated with increased risk of AD [1, 2] through mechanisms that include dyslipidemia, endothelial dysfunction, inflammation, and oxidative stress. In contrast, populations with greater consumption of fats as poly- or mono-unsaturated oils (PUFA and MUFA, resp.) have lower prevalence of AD and vascular based dementias [1–4], probably as a consequence of lower levels of systemic inflammation [5–7].

In AD, chronic inflammation leading to neuronal loss appears to be primarily associated with cerebrovascular and brain parenchymal deposits of amyloid beta (Aβ) [8]. Derived from the amyloid precursor protein, Aβ is the predominant component of "amyloid" (or senile) plaques [9, 10]. Key triggers of cerebrovascular amyloidosis are thought to include enhanced proteolytic processing of the precursor protein on the plasma membrane of neuronal cells [11–13], a phenomenon more common in early-onset AD. In addition, fibrillar formation of Aβ and deposition upon extracellular matrices may also reflect decreased degradation and efflux by epithelial cells of the choroid plexus [14, 15]. Alternatively, cerebral parenchymal Aβ load may be exacerbated if cerebrovascular integrity is compromised and

blood-to-brain delivery of peripheral Aβ is increased [16, 17]. Moreover, the latter typically results in the activation of astro-glial cells and oxidation of proteins and lipids [18, 19].

Significant plasma Aβ is found associated with triglyceride-rich lipoproteins (TRLs) and cell culture and immunohistochemical studies confirm secretion of Aβ as a TRL from hepatocytes and absorptive epithelial cells of the small intestine [20–22]. In humans there is a transient increase in plasma Aβ concentration, following the consumption of a mixed lipid meal and kinetic studies in vivo showing that Aβ serves as a regulating apolipoprotein of TRLs [23]. However, several lines of evidence suggest that persistent disturbances in the TRL-Aβ pathway may contribute to AD risk. In three strains of amyloid transgenic mice, secretion into plasma of TRL-Aβ was strongly associated with onset and progression of amyloidosis [24]. Moreover, significant cerebrovascular disturbances were reported preceding plaque formation in amyloid transgenic mice [25]. Consistent with the concept of disease induction in response to exaggerated exposure, subjects with AD were reported to have significantly elevated plasma TRL-Aβ concomitant with evidence postprandial dyslipidaemia [26]. Moreover, in human cadaver and in transgenic-amyloid mice brain specimens, significant apolipoprotein B (apo B) immunoreactivity colocalized with early diffused amyloid plaque [27, 28].

To explore directly the hypothesis of a dietary fat modulation of TRL-Aβ and cerebrovascular integrity axis, wild-type (WT) mice were fed diets enriched in either SFA, MUFA, or PUFAs [28]. Within 12 weeks of dietary intervention, mice maintained on the SFA diet showed substantial parenchymal extravasation of plasma proteins including apo B lipoproteins enriched with Aβ. The endothelial tight junction protein occludin was substantially attenuated in SFA-fed mice concomitant with substantially increased enterocytic abundance of Aβ [28, 29]. In contrast, mice maintained on either the MUFA or PUFA diets had no cerebrovascular aberrations and penetration of plasma proteins in these two groups was comparable to low-fat- (LF-) fed controls.

The potential cytotoxic properties of SFA are established and the principal mechanisms include mitochondrial respiratory burst resulting in oxidative stress and endoplasmic reticulum dysfunction [30]. Polyunsaturates on the other hand and in particular docosahexanoic acid (DHA) generally antagonise the effects of SFA and are purported to confer cytoprotection because of potent anti-inflammatory effects [5, 31–33]. However, unsaturated fatty acids such as DHA are highly susceptible to lipid peroxidation and if inflammation is already established, then oxidative damage may be paradoxically amplified with the provision of unsaturated fatty acids such as DHA. To explore the hypothesis that polyunsaturated fatty acids confer benefit and not risk in a cerebrovascular proinflammatory state, we explored parenchymal extravasation of apo B lipoproteins in WT mice initially maintained on an SFA-enriched diet for 3 months, followed by randomization to either an LF- or a PUFA-enriched diet for 2 months.

2. Methods

2.1. Animals and Diet Conditions. The animal housing, handling, and experimental procedures described for this study were approved by the Curtin University Animal Experimentation and Ethics Committee. Six-week-old female WT mice (C57BL/6J) were housed in groups and randomized into their respective diets ($n = 6$ mice per group). All mice were maintained on a 12 h light and dark cycle room, at 22°C and with free access to water and food.

Mice were fed an SFA diet (SF07-050, Glen Forrest Stockfeeders, Glen Forrest, Western Australia) for 3 months and then randomised to receive either an LF (AIN-93M, Glen Forrest Stockfeeders), PUFA-enriched (SF07-049, Glen Forrest Stockfeeders), or maintained on SFA for a further 2 months. Fatty acid composition for each diet is shown in Table 1. Digestible energy for the SFA diet was 18.8 MJ/kg and contained palmitic (16 : 0) and stearic (18 : 0) as the principle saturated fats (13%, w/w). The LF diet contained 4% (w/w) as total fat and 15.1 MJ/kg of digestible energy and only <1% of total digestible energy from lipids. The PUFA diet contained 8.22% (w/w) of DHA and 2.00% (w/w) eicosapentaenoic acid (EPA) sourced from NUMEGA fish oil. The diet generated 18.8 MJ/kg of digestible energy where 40% of energy derived from lipids. All diets had sufficient essential fatty acids.

2.2. Tissue Collection and Sample Preparation. Blood and brain samples were collected as previously described by Takechi et al. [28]. Mice were anaesthetised with pentobarbitone (45 mg/kg i.p.), and exsanguinated by cardiac puncture. Blood was collected into K-2 EDTA tubes and stored in ice. Plasma was separated by short speed centrifugation at 4°C and stored at −80°C.

Brain tissues were carefully isolated, washed with chilled phosphate buffer saline (PBS, pH 7.4), right hemispheres were separated, and fixed in 4% paraformaldehyde for 24 h. The tissues were then cryoprotected with 20% sucrose solution at 4°C for 72 h, frozen in isopentane with dry ice and stored at −80°C. For histology and fluorescence microscopy, serial cryosections of 18 μm were cut from the right cerebral hemispheres for each mouse and mounted on Polysine slides (LabServ, Australia).

2.3. Cerebral Apo B Immunofluorescence. Cerebrovascular leakage of apo B was detected as previously described [28]. Brain cryosections (18 μm) were air-dried for 30 min, rehydrated with PBS and incubated in blocking serum (10% goat serum) for 30 min prior to the application of the antibodies. Cerebral apo B was detected by overnight incubation with the primary antibody polyclonal rabbit anti-apo B (ab20737, Abcam, Cambridge, UK) at 1 : 500 dilution, at 4°C. Postovernight incubation and washing with PBS, primary antibody was labelled at room temperature with the secondary goat anti-rabbit IgG-Alexa 488 fluorochrome conjugate (A-11034, Invitrogen) for 2 h. The sections were then washed with PBS and the nuclei were counterstained with DAPI (1 : 1000) for 5 min at room temperature. Thereafter, the stained sections were mounted with antifade mounting

Table 1: Dietary composition.

	SFA diet	LF diet	DHA diet
Calculated nutritional parameters (%)			
Protein	13.6	13.6	13.6
Total fat	20.3	4	20.3
Crude fibre	4.7	4.7	4.7
Acid detergent Fibre	4.7	4.7	4.7
Digestible energy	18.8 MJ/kg	15.1 MJ/kg	18.8 MJ/kg
% Digestible energy from lipids	40	n/a	40
% Digestible energy from protein	15	n/a	15
Calculated fat composition (%)			
Myristic acid 14:0	0.05	Trace	0.54
Pentadecanoic acid 15:0	0.01	n/a	0.16
Palmitic acid 16:0	5.16	0.2	3.26
Megaric acid 17:0	0.05	n/a	0.18
Stearic acid 18:0	7.31	0.1	0.92
Arachidic acid 20:0	0.24	n/a	0.06
Behenic acid 22:0	0.04	n/a	0.05
Tetracosanoic acid 24:0	0.03	n/a	0.05
Palmitoleic acid 16:1	0.05	Trace	0.66
Heptadecenoic acid 17:1	0.01	n/a	0.10
Oleic acid 18:1 n9	6.62	2.4	2.25
Gadoleic acid 20:1	0.01	n/a	0.18
Lenoleic acid 18:2 n6	0.67	0.8	0.23
a Linolenic acid 18:3 n3	0.05	0.4	0.09
g Linolenic acid 18:3 n6	Not detected	n/a	0.08
Arachadonic acid 20:4 n6	Not detected	Trace	0.46
EPA 20:5 n3	Not detected	Trace	2.00
DHA 22:6 n3	Not detected	Trace	8.22

The total fatty acid composition of SFA, LF, and DHA diets. Vitamin and mineral contents were balanced in all diets.

medium. Primary antibody was replaced with buffer or an irrelevant serum for negative control tissues.

2.4. Quantitative Immunofluorescent Imaging and Analysis. Digital images for photomicroscopy were acquired through AxioCam HRm camera (Carl Zeiss, Germany) with an AxioVert 200 M inverted microscope by Zeiss (Germany) at ×200 magnification (Plan Neofluar ×20 objective, 1.3 numerical aperture). Three-dimensional (3D) images were captured through ApoTome optical sectioning methodology (Carl Zeiss). Each 3D image consisted of 6–10 two-dimensional (2D) images and the distance between Z-stack slices was 1.225 μm optimised by Nyquist. A minimum of nine 3D images were randomly captured per mouse, which include 5 images within the cortex (CTX) and 2 images each from brainstem (BS) and hippocampal formation (HPF).

Cerebrovascular leakage of plasma protein apo B was quantified within the CTX excluding the hippocampus, BS, and HPF. The pixel intensity of the protein of interest for each 3D image was calculated utilising the automated optical intensity measurement tool in Volocity (Software version 5.5, Perkin Elmer, Melbourne, Australia) and expressed as per unit volume. The investigator was blinded during imaging and quantitation.

2.5. Plasma Cholesterol, Triglyceride, and NEFA. Plasma cholesterol and triglycerides were determined in duplicate by enzymatic assays (Randox Laboratories LTD, UK). Non-esterified fatty acids (NEFAs) were determined with NEFA-C (ASC-ACOD method, Wako Pure Chemical Industries, Osaka, Japan).

2.6. Plasma S100β Analysis. Plasma S100β is used as a marker of cerebrovascular inflammation and was measured using ELISA kits according to manufacturers' instructions (CosmoBio, Tokyo, Japan). Plasma S100β was measured with 30 μL of plasma sample or standard and incubated in precoated microtitre well plates at 4°C overnight. Plates were then incubated with biotinylated secondary antibody and Streptavidin-HRP for 2 h, each. Colour generated with substrate and optical density determined at 492 nm. After adjusting for sample dilution, final concentrations of plasma S100β were extrapolated from standard curve.

2.7. Statistical Analysis. This study utilised $n = 6$ mice per group and minimum of nine 3D images were captured per mouse for detection of apo B leakage. Each 3D image was constructed by stacking of sequential 2D images, therefore generating 324–540 two-dimensional images per group. All

Table 2: Effects of various feeding regimens on plasma lipids in wild-type (C57BL/6J) mice.

	SFA 5 m	+LF 2 m	+DHA 2 m
TG (mM)	0.39 ± 0.04	0.54 ± 0.03*	0.43 ± 0.04
TC (mM)	1.45 ± 0.23	1.69 ± 0.17	3.11 ± 0.12**
NEFA (mEq/L)	0.42 ± 0.03	0.45 ± 0.03	0.53 ± 0.05
Body weight			
Final	25.65 ± 0.64	23.72 ± 0.96	20.21 ± 0.50**
Weight gain	8.41 ± 0.51	5.7 ± 1.12	3.3 ± 0.51**

Plasma total cholesterol (TC), triglycerides (TGs), and non-esterified fatty acids (NEFAs) were measured at the end of the feeding regimen in mice fed saturated fats (SFA 5 m) and SFA-fed mice switched to an LF (+LF 2 m) and DHA diet (+DHA 2 m). Final body weight and weight gain were also calculated. Data represented as mean ± standard error of mean. Means were compared with one-way ANOVA, where $P < 0.05$ considered statistically significant (*).

data was analysed by either parametric or nonparametric one-way Analysis of Variance to assess the main effects of LF and DHA on dietary SFA-induced blood-brain barrier (BBB) dysfunction and their two-way interactions. Post-hoc comparison of means was done if the associated main effect or interaction was statistically significant within the Analysis of Variance procedure. P values < 0.05 were considered statistically significant.

3. Results

We confirm previous studies showing significant CTX > BS > HPF extravasation of apo B lipoproteins in WT mice maintained on an SFA diet for a total of 5 months (3 months plus randomization to SFA for an additional 2 months: Figures 1 and 2). Mice randomized to an LF diet following 3 months of SFA feeding had comparable levels of parenchymal apo B lipoprotein abundance to mice maintained on SFA feeding alone (Figures 1 and 2). However, in mice randomized to the DHA-enriched diet following 3 months of SFA feeding, parenchymal apo B abundance was markedly increased (Figures 1 and 2). The cerebrovascular effects of the DHA diet occurred commensurate with a 2-fold increase in plasma cholesterol compared to mice maintained on the SFA diet (Table 2). In contrast, the LF diet had no significant impact on plasma lipid homeostasis. Disturbances in BBB integrity and function were supported by the findings of substantially increased plasma S100β in the SFA → DHA mice compared to SFA alone (Figure 3). The protein S100β is commonly used as a surrogate marker of brain-to-blood leakage. The S100β is a cytokine produced exclusively by the astrocytes of the central nervous system. Following randomization, differences in food consumption were identified. Mice maintained on SFA or randomized to the LF consumed on average 3 g/day, whereas consumption of the DHA-enriched diet was reduced to 2 g/day. The lower caloric intake of mice on the DHA enriched diet resulted in a slower rate of growth following randomization (Table 2).

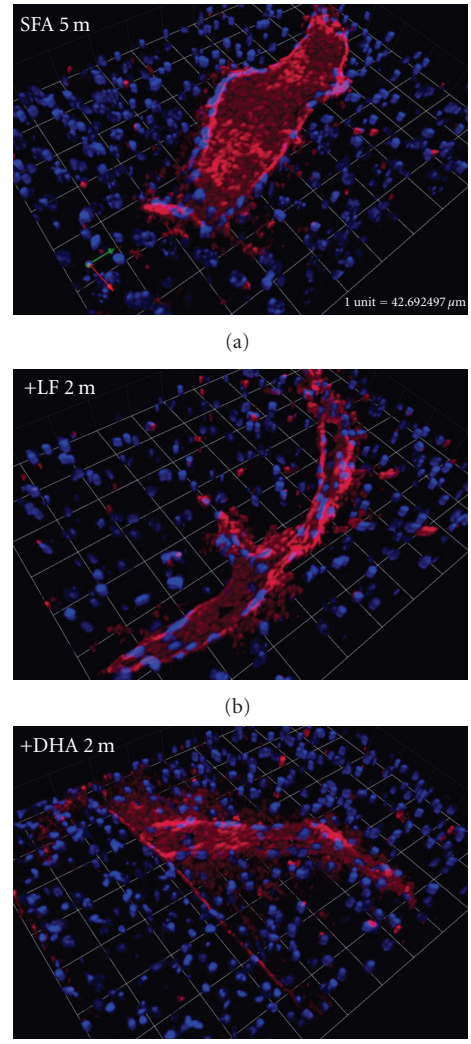

Figure 1: Three-D immunofluorescent staining of cerebral extravasation of apo B. Parenchymal leakage of apo B lipoproteins (red) is observed surrounding the cerebral microvessels. Nuclei are shown in blue. The 3D images are from mice maintained on saturated-fat diet for 5 months (SFA 5 m) and SFA fed mice randomised to LF (+LF 2 m) and DHA (+DHA 2 m) diet for further 2 months. Scale: 1 unit = 42.7 μm.

4. Discussion

This study was designed to explore if provision of a diet enriched in DHA attenuated cerebrovascular dysfunction induced by chronic ingestion of an SFA diet. The primary outcome measure was to determine the abundance of brain parenchymal apo B lipoproteins that transport significant endogenous Aβ. Cerebral capillary vessels normally have tightly apposed endothelial cells that ordinarily prevent transport of plasma proteins and macromolecules [34].

The primary finding of this study showed that provision of a PUFA diet principally enriched in DHA exacerbated brain parenchymal extravasation of apo B lipoproteins that had been initially induced by chronic ingestion of SFA. Previous studies exploring the effect on cerebrovascular integrity

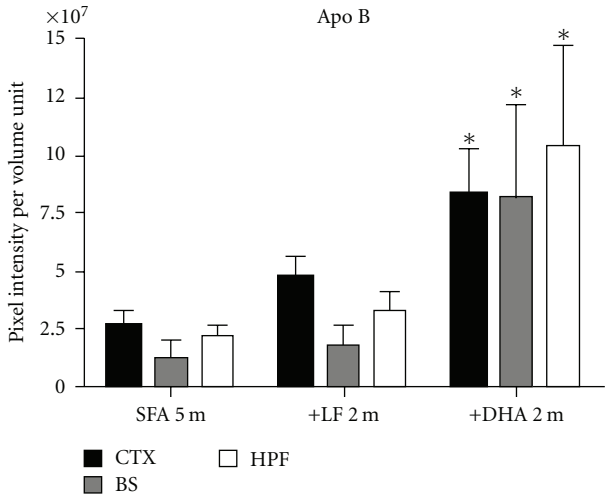

Figure 2: Three-dimensional (3D) quantitative analysis of apo B leakage in C57BL/6J mouse brain. Pixel intensity of apo B lipoproteins surrounding the cerebrovasculature was quantitated in 3D images from mice maintained on saturated-fat diet for 5 months (SFA 5 m) and SFA-fed mice randomised to LF (+LF 2 m) and DHA (+DHA 2 m) diet for further 2 months. The extent of apo B pixel intensity was measured in the cortex (CTX), brain stem (BS), and hippocampal formation (HPF) and expressed as per unit volume. The bars represent mean intensity and standard error of mean, where $P < 0.05$ considered statistically significant (* Kruskal Wallis t-test). The C57BL/6J mice randomised to DHA diet (+DHA) had elevated apo B intensities in all regions of the brain.

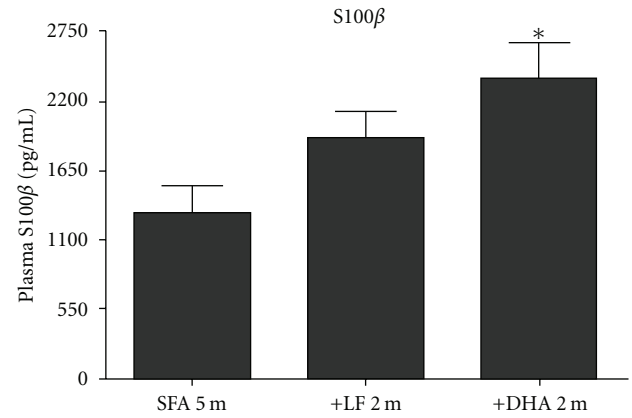

Figure 3: Effect of dietary fatty acids on plasma S100β concentration in C57BL/6J mice. The brain abundant protein S100β was measured in plasma as a surrogate marker of BBB leakage. High plasma S100β concentration in SFA-fed mice correlate with significant BBB dysfunction. In comparison to SFA 5 m group, plasma S100β was significantly increased in mice switched to the DHA diet (+DHA 2 m). The bars represent mean plasma concentration (pg/mL) and standard error of mean, where $P < 0.05$ considered statistically significant (* one-way ANOVA).

and function by the SFA and PUFA diets described here, as well as an MUFA-enriched diet, showed in C57BL/6J mice that only the SFA diet induced parenchymal accumulation of apo B lipoproteins [28]. Therefore, the paradoxical effects of the PUFA diet are likely to be a consequence of amplification of proinflammatory pathways induced as a consequence of chronic SFA ingestion. Consistent with this concept, SFA-fed mice randomized to an LF diet showed similar parenchymal apo B abundance and plasma S100β as mice that were maintained on SFA alone.

Several studies have provided evidence of a vasoactive role of Aβ, with pathological manifestations prior to Aβ deposition. Exogenous administration of Aβ is vasoconstrictive and vessels treated with Aβ show significant endothelial cell damage with changes in the cell membrane, cytoplasm, nucleus, and other organelles [16]. Takechi et al. [28] suggested that that chronic ingestion of SFA may increase TRL-Aβ secretion and that repeated postprandial excursions may eventually disrupt BBB function. Consistent with this possibility, SFAs were shown to stimulate enterocytic Aβ abundance and released into the circulation associated with postprandial TRL and a similar phenomenon may also occur in liver [20, 35]. Moreover, diets enriched in SFA reduce high affinity clearance pathways of TRL-remnant lipoproteins and this may contribute to increased postprandial lipaemia and plasma Aβ [36, 37].

In this study, parenchymal apo B abundance did not correlate with plasma triglyceride concentration. Mice fed the DHA-enriched diet had comparable triglycerides to the SFA group and plasma triglycerides were greatest in SFA mice randomized to LF. Saturated fats often increase plasma NEFA concentration compared to low-fat diets, whereas DHA generally accelerates TRL clearance by facilitating LPL-mediated lipolysis [38, 39]. However, in this study, there was no significant increase in net concentration of plasma NEFA in mice randomized to the DHA diet versus those maintained on SFA alone (0.53 ± 0.05 versus 0.42 ± 0.03 mEq/L, resp.). Nonetheless, a role of fatty acids in modulating cerebrovascular integrity cannot be ruled out because fatty acid phenotype may be critically important. Many studies suggest significant vascular effects of fatty acids. Human aortic endothelial cells treated with TRL and lipoprotein lipase (LPL) were highly permeable, whilst cells treated with TRL or LPL alone were not [40]. Furthermore, LPL-mediated TRL lipolysis initiated degradation of the tight junction protein ZO-1 and induced an endothelial apoptotic cascade.

The most significant lipid effect of randomization to DHA following chronic ingestion of SFA in this study was a twofold increase in plasma cholesterol compared to the mice maintained on the SFA diet, or mice randomied to the LF diet. A number of animal and clinical studies have shown that DHA-enriched diets can increase plasma cholesterol associated with low- and high-density lipoproteins [41–43]. Hypercholesterolemia has been associated with many vasculature abnormalities including endothelial dysfunction, decreased vascular reactivity, and enhanced expression of adhesion molecules [44, 45]. Cell culture studies suggest several mechanisms by which cholesterol may be pro-inflammatory and some of these appear to be analogous to the effects of dietary SFA. Yao and colleagues reported that excess cholesterol causes endoplasmic reticulum and mitochondrial stress that can lead to apoptosis [46]. Mitochondrial activity or microsomal processing also results in the production

of oxidized lipids that trigger and exacerbate inflammatory pathways [47].

The anti-inflammatory properties of particularly the n3 and n6 fatty acids have been unequivocally demonstrated in many studies. However, the propensity for PUFA to oxidize may under some circumstances amplify oxidative stress sequelae. Diets enriched in SFA enhance oxidation as a consequence of stimulated mitochondrial function in activated macrophages [46]. Dietary SFA diminish the proper function of the cerebrovascular endothelial cells and are thereafter likely to activate astro-glial cells which encompass cerebral capillary vessels. It's plausible that enhanced interaction of plasma PUFA's in DHA-fed mice with activated inflammatory cells may be a primary mechanism by which the effects of SFA are amplified. Consistent with this possibility, Kuo et al. [48] showed a dose effect of dietary DHA on BBB permeability in mice supplemented with 12% fish oil for 6 months, versus mice fed 3% fish oil. Similarly, rats consuming fish oil exhibited increased lipid peroxidation [49, 50] and oxidative stress-induced damage of DNA in the absence of dietary antioxidants [51]. In vitro, DHA and EPA enhanced lipid peroxidation and triggered cellular apoptosis [52, 53].

5. Summary

Chronic ingestion of diets enriched in SFA commonly causes vascular dysfunction, including in capillary vessels of the brain. The effects of SFA could be described as a response-to-injury phenomenon induced by exaggerated exposure to plasma triglyceride, cholesterol, NEFA, or harmful inflammatory products of lipid metabolism, such as lipid peroxides. Many studies support a role of n3 and n6 fatty acids in the prevention of vascular, based disorders primarily via suppression of inflammatory cascades. Less clear however are the benefits of polyunsaturated oils in the presence of profound inflammation, because of the propensity to generate lipid peroxidation products.

In an established model of cerebrovascular dysfunction induced by chronic ingestion of an SFA-enriched diet, provision of DHA amplified the harmful effects. Probable mechanisms include hypercholesterolemia and perhaps fatty acid-induced cytotoxicity. The data suggests that introduction of n3/n6 fatty acids in metabolic conditions that are characterized by heightened systemic inflammation needs to be carefully considered in the context of paradoxical detrimental effects that could occur.

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Review Article

The Chylomicron: Relationship to Atherosclerosis

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The B-containing lipoproteins are the transporters of cholesterol, and the evidence suggests that the apo B48-containing postprandial chylomicron particles and the triglyceride-rich very low density lipoprotein (VLDL) particles play an important part in the development of the plaque both directly and indirectly by their impact on LDL composition. The ratio of dietary to synthesised cholesterol is variable but tightly regulated: hence intervention with diet at best reduces serum cholesterol by <20% andusually <10%. Statins are the mainstay of cholesterol reduction therapy, but they increase cholesterol absorption, an example of the relationship between synthesis and absorption. Inhibition of cholesterol absorption with Ezetimibe, an inhibitor of Niemann Pick C1-like 1 (NPC1-L1), the major regulator of cholesterol absorption, increases cholesterol synthesis and hence the value of adding an inhibitor of cholesterol absorption to an inhibitor of cholesterol synthesis. Apo B48, the structural protein of the chylomicron particle, is synthesised in abundance so that the release of these particles is dependent on the amount of cholesterol and triglyceride available in the intestine. This paper will discuss cholesterol absorption and synthesis, chylomicron formation, and the effect of postprandial lipoproteins on factors involved in atherosclerosis.

1. Introduction

The formation of the chylomicron is complex. In healthy humans virtually all fat is absorbed, but cholesterol is tightly regulated depending on body needs. A very flexible mechanism has evolved to keep serum cholesterol in a very narrow range. The chylomicron is responsible for the transport of medium- and long-chain fatty acids, together with cholesterol into the lymph. Apo B 48, the solubilising protein for the chylomicron, is secreted by the entrecote. Unused protein is degraded, a mechanism that ensures that there is sufficient apo B48 for even the largest fat meal. Fat feeding increases apo AIV expression, and apo A1V serves as a surface component for apo B48 particles in the entrecote [1, 2]. Apo A IV may stimulate net transfer of membrane triglyceride to luminal particles. It has been suggested that this occurs due to an increase in microsomal triglyceride transfer protein (MTP) at the pretranslational level [3]. The chylomicron particle comes in many sizes; thus the excessive fat load may be carried by an increase in particle numbers and/or size. Chylomicron assembly may involve 3 major processes: assembly of primordial lipoproteins, formation of lipid droplets, and core expansion [4].

2. The Chylomicron

The chylomicron is assembled mainly in the ER and is then transported to the *cis*-golgi in prechylomicron transport vesicles (PCVs) [5–7]. The secretion of the particles from the intestine is regulated by MTP. Among the functions of MTP is the ability to initiate the incorporation of lipids into apo B preventing apo B degradation. MTP acts as a chaperone to assist in apo B folding [8] (For excellent review of intestinal lipid absorption see Iqbal and Hussain [9]). Inhibitors of MTP in the intestine cause steatorrhoea are associated with weight loss and a reduction in cholesterol and triglycerides in human studies [10].

The chylomicron may be thought of as a particle that has as its main function the transport of fat from the intestinal lumen to the liver. On the way to uptake by the liver, the chylomicron divests itself of lipid to the adipocyte for storage or the muscle and other cells for energy. It also carries

cholesterol which is made available following the controlled absorption under the regulation of NPC1L1 and ATP binding cassette proteins (ABC) G5/G8. Chylomicron cholesterol is also available from the enterohepatic circulation and from de novo synthesis by the enterocyte. To study the chylomicron particle formation it is interesting to examine the effects of insulin resistance, a condition associated with an increase in chylomicrons. The fructose-fed hamster is a model of diet-induced insulin resistance. Wong et al. [11] examined the proteomic profiles of prechylomicron transport vehicles (PCVs) isolated from the enteric endoplasmic reticulum in the small intestine. They found a number of PCV-associated proteins to be differentially expressed in these animals including MTP, apo B48, Sar-1, and vesicle-associated membrane protein 7 (VAMP-7). Glucagon-like peptide-2 (GLP-2), a gastrointestinally derived intestinotropic hormone that links nutrient absorption to intestinal structure and function, was administered to hamsters and was found to increase secretion of apo B48, triglyceride-rich lipoprotein (TRL), and cholesterol mass. GLP 2 directly stimulated apo B48 secretion in jejunal fragments cultured ex vivo [12]. They further suggest that GLP2 represents a nutrient signal that regulates intestinal absorption of lipid and the assembly and secretion of chylomicrons from intestinal enterocytes. CD36 is a member of the class B scavenger receptor family of cell surface proteins, and the ability of GLP2 to increase intestinal lipoprotein production was lost in the CD36 $-/-$ mice suggesting that the mechanism of action of GLP2 is through CD 36.

To examine how cholesterol might influence the chylomicron it is interesting to consider the impact of plant sterols which lower intestinal absorption of cholesterol. Amiot et al. [13] have recently shown that plant sterol esters reduced meal-derived labeled cholesterol in chylomicrons but did not alter triglyceride hydrolysis or change chylomicron lipids nor the time course of postprandial chylomicron lipid increase. Ezetimibe inhibits NPC1L1 by binding to the protein and preventing conformational changes necessary for translocation of cholesterol across the membrane [14]. Bozzetto et al. [15] examined the role of Ezetimibe both fasting and after a standard meal in type 2 diabetic patients. They found that Ezetimibe added to a statin significantly decreased chylomicron cholesterol and triglyceride concentrations and postprandial apo B48. Masuda et al. [16] have shown that Ezetimibe inhibits postprandial hyperlipidaemia in patients with Type 2b hyperlipidaemia. CD 36 deficient mice have enhanced synthesis of chylomicrons in the small intestine. Sandoval et al. [17] showed that Ezetimibe reduces postprandial hyperlipidaemia in both wild-type and CD36 KO mice. They showed that triglyceride content and apo B48 mass were decreased and intestinal mucosal mRNA expression of fatty acid transfer protein 4 and apo B, along with fatty acid binding protein 2 (FAB2), diacylglycerol O-acyltransferase (DGAT)-1 and -2, and stearoyl-CoA desaturase (SCD)-1 which is involved in the synthesis and regulation of unsaturated fatty acids, were downregulated. It seems therefore that Ezetimibe has more actions than just down regulating cholesterol absorption, and these studies may help in evaluating the role of cholesterol in chylomicron assembly. It is particularly difficult to understand how triglyceride absorption is altered since Ezetimibe has not been shown to cause weight loss or steatorrhoea. Turnover studies might help to understand the role of NPC1-L1 in fat metabolism. It is possible that increased chylomicron particle clearance, due to the smaller load, plays a part in the above results.

3. Niemann Pick C1-Like1

The discovery of Niemann Pick C1-like1 (NPC1L1) is an interesting story. The search for an explanation as to how cholesterol in the body is so finely regulated has been intensive. The finely tuned regulation of cholesterol was perhaps best illustrated by the report of the elderly gentleman who ate 25 eggs a day for many years but his cholesterol remained at just above 6 mmol/L [18]. Altman and Davis in their search for molecules that might inhibit cholesterol absorption discovered by chance a compound which is now known as Ezetimibe [19]. They discovered a putative gene, the Niemann Pick C1-like1 (NPC1-L1). Elegant studies in mice demonstrated that knocking out this gene reduced cholesterol absorption by the same amount as happened when the wild mice were fed with Ezetimibe. They showed that there was no further reduction in cholesterol absorption in the knockout mice when fed Ezetimibe. The group went on to show that lack of NPC1L1 in apoE$^{-/-}$ mice results in a significant reduction in cholesterol absorption and plasma cholesterol levels and caused a nearly complete protection from the development of atherosclerosis, under both cholesterol-fed and non-cholesterol-fed conditions [20, 21]. Weinglass et al. [14] have shown that Ezetimibe binds to a site distinct to the site where cholesterol binds, preventing conformational changes in NPC1L1 that is necessary for translocation of cholesterol across the membrane. Statins, which inhibit HMGCoA reductase and cholesterol synthesis, have been shown to increase cholesterol absorption. It has also been shown that low absorbers of cholesterol respond better to statins than high absorbers [22]. Ezetimibe potentiates the effect of statins, increasing their effectiveness by another 15–20% in relation to cholesterol lowering. Tremblay et al. [23] reported an increase in NPC1L1 by 19% on atorvastatin thus describing a mechanism whereby cholesterol absorption is increased in patients on statins. The mechanism of action of NPC1L1 has recently been further elucidated. It has been shown that cholesterol promotes the formation and endocytosis of NPC1L1 which appears to be an early step in cholesterol uptake. Zhang et al. [24] have discovered that it is the N-terminal domain of NPC1L1 that binds cholesterol. It is interesting that this domain does not bind to plant sterols; thus it now seems that plasma membrane-bound NPC21L1 binds exogenous cholesterol and this binding facilitates the formation of NPC1L1-flotiln-cholestrerol microdomains that are then internalized into cells through the clathrin AP2 pathway. In animal studies we have demonstrated an increase in cholesterol absorption in diabetes [25]; we asked the question as to whether diabetes might be associated with an increase in cholesterol absorption through stimulation of NPC1-L1. We demonstrated in animal models of diabetes that NPC1L1 was upregulated [26]

and in diabetic patients, and we demonstrated an increase in NPC1L1 mRNA [27] suggesting a mechanism for an increase in cholesterol absorption. In the Psammomys Obesus, a model of type 2 diabetes, the animals exhibiting weight gain, hyperinsulinaemia, and hypercholesterolaemia, NPC1-L1 protein and gene expression were both significantly reduced in the intestine, and the authors found a lower capacity to absorb cholesterol compared to controls [28]. This may suggest interspecies variation but it is a surprising finding considering that this animal model of diabetes has been shown to have increased production of intestinal lipoprotein-containing apo B48 [29]. Ezetimibe has been shown to bind to the brush border and to NPCILI-expressing cells, [30]. There is a sterol regulatory element in the promoter and a sterol-sensing domain of NPCILI which appears to regulate cholesterol absorption in response to cholesterol intake. Huff et al. [31] have shown that NPC1L1 is suppressed in mice given a cholesterol-rich diet and increased in the cholesterol-depleted porcine intestine. The nuclear receptor, peroxisome proliferator-activated receptor (PPAR) delta/beta, appears to control the expression of NPC1L1. Activation by a synthetic agonist of PPAR delta has been shown to reduce cholesterol absorption and reduce expression of NPC1L1 without altering ABC G5/8 [32]. Tremblay et al. [23] have shown that Atorvastatin increases Niemann Pick C1L1 in the intestine and decreased ABC G 5/8 which leads to an increase in cholesterol absorption. These findings were accompanied by an increase in the transcription factors, sterol regulatory binding protein (SREBP) 2 and hepatic nuclear factor (HNF)-4. There may be other transporters of cholesterol, for example, scavenger receptor class B type 1 (SR-B1) [33] which is located both in the apical and basolateral membranes of the enterocyte. They may also play a role in cholesterol absorption. (For review see Iqbal and Hussein [9]).

4. ATP Binding Cassette Proteins G5 and G8

The mechanism whereby the body is almost completely unable to absorb plant sterols was a mystery until recently. Study of the familial condition, sitosterolaemia, unlocked the mystery [34]. Sitosterolaemia is a rare condition associated with early and severe atherosclerosis. The condition is associated with normal or slightly elevated cholesterol whereas total sterols are markedly increased. Search for polymorphisms in putative genes, controlling plant sterol absorption or perhaps one should say blocking plant sterol absorption, identified ATP binding cassette proteins (ABC) G5 and G8 in the intestine [35]. Further work demonstrated that these two gene products work in tandem to reexcrete both plant sterols virtually completely and cholesterol to a lesser extent in a regulated way [36]. The genes were also found to be expressed in the liver where they are responsible for controlling cholesterol reexcretion into the bile [37]. It appears that these two genes are very important regulators of cholesterol and together with NPC1-L1 protein are responsible for cholesterol homeostasis in the body.

Polymorphisms of the ABCG5/G8 have not only been associated with increased sitosterol but also with increase in cholesterol. It has also been shown that polymorphisms in the ABCG5/G8 may influence cholesterol in weight reduction programs (the Q604E SNP in ABCG5 and the C54Y in ABC8) [38]. Gylling et al. [39] examined polymorphisms in the ABCG5 and G8 genes and found that low serum cholesterol and cholesterol absorption were linked to a polymorphism (D19H) of the ABCG8 gene and characteristics of the insulin resistance syndrome in men were linked to Q604E polymorphism in the ABCG5 gene. The authors studied 263 mildly hypercholesterolaemic noncoronary subjects using cholestanol to cholesterol ratio as a surrogate marker of cholesterol absorption efficiency.

Since diabetes is so frequently associated with dyslipidaemia and atherosclerosis, the ABCs became a target for research. Bloks et al. [40] examined mRNA and protein expression of ABCG5 and G8 in the intestine of streptozotosin diabetic rats and found significant reduction in expression of both ABCG5 and G8. They found that levels were partially normalised on insulin supplementation. We have shown that ABCG5 and G8 were reduced by more than 50% in the intestine of zucker diabetic fa/fa rats compared with lean rats although this did not reach statistical significance [41]. Insulin treatment caused a non-significant increase in ABCG5 and G8 mRNA. In another study of streptozotosin diabetic rats ABCG5 and G8 were both very significantly reduced in the intestine [26]. There was a negative correlation between ABCG5 and G8 and chylomicron cholesterol [26]. In the Psammomys Obesus, another model of diabetes, Levy et al. [28, 29] showed a reduction in ABC G5/G8 in the intestine. In the intestine of human subjects with type 2 diabetes, ABCG5 and G8 mRNA were both significantly lower compared to controls [27]. There was a negative correlation between ABCG5 and G8 and NCP1-L1 in the combined diabetic and control subjects [27]. There was a significant negative correlation between chylomicron cholesterol and both ABCG5 and G8 [27]. These two genes appear to play an important role in the dysregulation of cholesterol metabolism in diabetes.

As stated above inhibition of HMG CoA reductase with a statin has been shown to decrease ABC G5/8 as well as increasing NCP1L1 to increase cholesterol absorption [23]. In the Psammomys obesus animal model of type 2 diabetes the ABC G5 /8 reduction was associated with a reduction rather than increase in cholesterol absorption perhaps due to a reduction in NPC1L1 which the authors found, suggesting a difference between different animal models of diabetes [28].

Calcium appears to regulate lipids, at least in post menopausal women where supplementation has been shown to favorably alter lipids. Ma et al. [42] have shown in hamsters that improvement was associated with downregulation of NPC1L1 and MTP mRNA and upregulation of intestinal ABCG5/8.

5. Intestinal Microsomal Triglyceride Transfer Protein

Intestinal microsomal triglyceride transfer protein (MTP) plays a major role in the "assembly of the chylomicron particle and therefore of cholesterol and triglyceride metabolism.

MTP has become a hot topic since inhibitors of intestinal MTP have been shown to lower triglyceride without causing hepatic steatosis at least in animal studies [43–45]. Although many polymorphisms of MTP have been described, some of which have considerable impact on LDL cholesterol in both nondiabetic and diabetic subjects [46, 47], it is difficult to know whether the results mainly stemmed from the effect in the liver rather than the intestine. The intestinal inhibitors of MTP which have no effect on the liver should answer this question in the future. In animal studies, diabetes is associated with an increase in MTP mRNA with close correlation between MTP mRNA and chylomicron cholesterol [40, 41, 48, 49]. In the rabbit increased intestinal MTP mRNA is associated with increase in chylomicron particle numbers [48], but in the rat it is associated with larger particles [49]. The fructose-fed insulin-resistant hamster model had an increase in MTP protein mass, and this was associated with an increase in the triglyceride-rich intestinally derived lipoproteins [50]. Zoltowska et al. in 2003 [51] examined the B48-containing lipoprotein assembly in the small intestine of *Psammomys obesus*, a model of nutritionally induced diabetes and insulin resistance. De novo triglyceride synthesis, apo B48 biogenesis, and triglyceride-rich lipoprotein assembly were all increased. MTP activity and protein expression, however, were not altered. In the enterocyte of fructose-fed golden hamster MTP mRNA and protein mass were increased by TNFα, but apo B levels in the enterocyte were not affected suggesting that there is considerable inter species variation [50]. In human studies in type 2 diabetes we demonstrated an increase in MTP mRNA in intestinal biopsies [27, 46]. Diabetic patients who were on statin therapy had lower MTP mRNA compared to those not on statins [46]. We found positive correlations between MTP mRNA and chylomicron fraction cholesterol and apo B48 [46].

6. MTP Polymorphism

In type 2 diabetes the common MTP 492 G/T polymorphism has been associated with surrogate markers of nonalcoholic hepatic steatosis [52]. The homozygous form of the polymorphism has been shown to be associated with a higher concentration of LDL 3 in diabetic patients of Chinese origin, but there was no effect in the heterozygous subjects [53]. In nondiabetic subjects heterozygous for the T allele no changes in LDL triglyceride or cholesterol have been found whereas the few subjects homozygous for the T allele had decreased numbers of triglyceride-rich VLDL particles and significantly lower VLDL and LDL cholesterol [54]. It has been suggested that the T allele might interact with visceral obesity and hyperinsulinaemia in nondiabetic subjects [55]. Examination of fasting lipids in a healthy black male population demonstrated that the rare T/T genotype was associated with a higher mean level of apo B suggesting a racial difference [56]. The effect of the influence of the G/T polymorphism on postprandial lipoproteins demonstrated that the TT polymorphism was associated with an increase in apo B48 in the smallest triglyceride-rich lipoprotein fraction postprandially without a difference in postprandial triglycerides or in fasting plasma or TRL cholesterol [54]. We have found in diabetic patients that the heterozygous −493 G/T polymorphism was associated with significantly lower LDL cholesterol and in the postprandial period higher apo B48 in the small chylomicrons [46]. It was a surprise to us that the −493 G/T homozygous subjects who had lower LDL cholesterol were reported to have an increase in CHD [57]. Homozygosity of the minor −493 T allele has been associated with an increased risk of IHD in 2 further studies [58, 59]. These intriguing findings have been further investigated by Aminoff et al. [60]. Aminoff et al. [60] showed that both the MTP polymorphisms −493 G > T and the 164 T > C result in lower transcription of MTP in vivo in the heart, liver, and macrophage. They showed in a case-controlled study that the subjects homozygous for −164 C allele had an increased risk of IHD. These studies, together with the knowledge that the heart secretes Apo B-containing lipoproteins, suggest that reduction in MTP in the heart results in lipid accumulation in the heart and is followed by IHD or the susceptibility to IHD perhaps through reduction in availability of free fatty acids for energy at times of acute stress. Of course another theory is that just like the liver the decrease in secretion of VLDL through reduction in MTP function that leads to hepatic steatosis in the heart leads to accumulation of fat that may be toxic to the myocardium. Last year Bharadwaj et al. [61] demonstrated that in an animal model VLDL and chylomicron lipids enter the heart through different pathways. A CD36 process appears to be important for VLDL lipoproteins and a non-CD36 for chylomicron-derived fatty acid uptake. They showed that lypolysis is involved in the uptake of core lipids from triglyceride-rich lipoproteins. AMPK plays a central role in energy homeostasis. In the heart it increases during ischaemia and is thought to be implicated in the pathophysiology of cardiovascular and metabolic disease. AMPK has been targeted as being of value in the production of new therapies for cardiac and metabolic disease. AMPK is insulin sensitive, and in diabetes a reduction in AMPK activity leads to a decrease in muscle glucose uptake thus shifting fuel from glucose to fat for cardiac myocyte function. It is therefore interesting to speculate that the −493 polymorphism might be particularly a disadvantage to patients with diabetes. A further recent link to the possible importance of MTP activity in the cardiac muscle in diabetes is the finding that the redox-sensitive transcription factor NF-E2-related factor 2 (NrF2) is suppressed by extracellular signaling-related kinase (ERK) leading to an increase in stress-induced insulin resistance in cardiac myocytes [62]. The authors also showed in the hearts of streptozotosin-induced diabetic mice downregulation of glucose utilization. These studies demonstrate the importance of the chylomicron and MTP in cardiac function/dysfunction and may account at least to some extent for the worse prognosis in those diabetic patients who have a MI.

7. Regulation of Chylomicron Synthesis

The synthesis of triglyceride in the liver depends on acyl-coenzyme-A-diacylglycerol acyltransferase (DGAT). This en-zyme is also found in many other tissues including

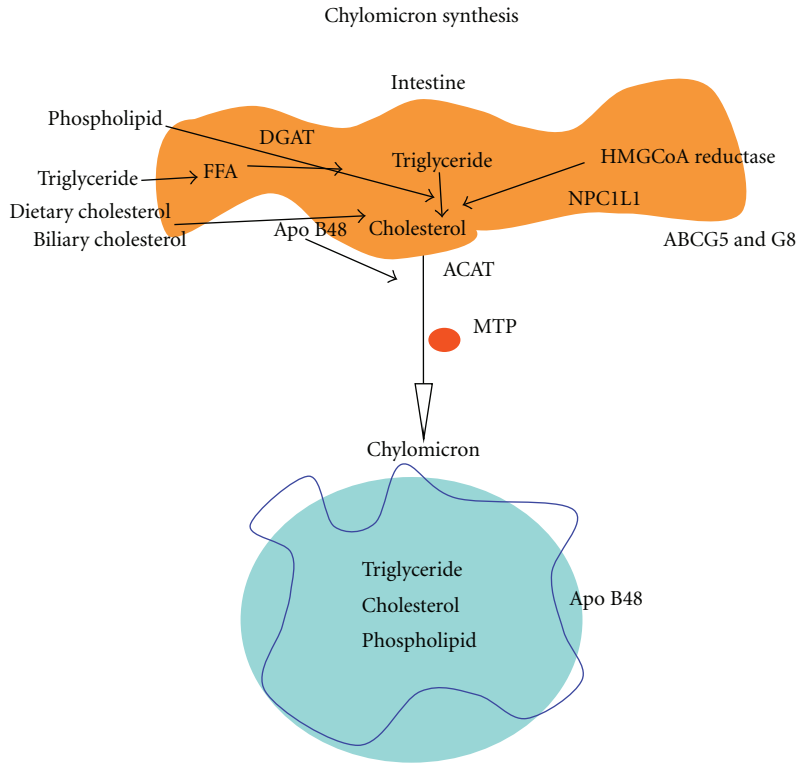

Figure 1: chylomicron synthesis. Dietary triglyceride, phospholipid, and cholesterol, together with intestinally synthesized cholesterol (for which 3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase is the rate-limiting enzyme) and recycled biliary cholesterol together with intestinally derived ApoB48 are assembled under the influence of microsomal triglyceride transfer protein (MTP) to form the chylomicron. Prior to incorporation into the chylomicron, cholesterol is esterified by acylcoenzyme A:cholesterol acyltransferase (ACAT) and the triglycerides are reassembled from fatty acids with acylcoenzyme-A-diacylglycerol acyltransferase (DGAT) catalysing the rate-limiting step. The cholesterol is transferred across the membrane by Niemann Pick C1 like-1 and some is reexcreted back into the intestinal lumen by the action of ATP-binding cassette proteins (ABC)G5 and -G8. The particle is assembled under the regulation of microsomal triglyceride transfer protein and delivered to the lymphatics.

the intestine and white adipose tissue, tissues that are active in triglyceride syntheis. Two DGAT enzymes have been discovered, DGAT1 and DGAT 2. These enzymes catalyse the final step of the triglyceride pathway [63], their substrates being diacyl glycerol and fatty acyl CoA. DGAT-1 deficient mice are resistant to diet-induced obesity and have increased sensitivity to insulin and leptin [64, 65], hence the excitment in discovering DGAT inhibitors [66, 67]. DGAT stimulates PPARs, and PPARa regulates lipid metabolism through its affect on adipocyte formation. Activation of PPARa with Fibrates lowers triglyceride through a number of mechanisms including increasing free fatty acid β-oxidation, hepatic lipoprotein lipase expression, a reduction in apo C111, and a reduction in apo B [68]. The mechanism by which PPARα activation represses apoCIII transcription has yet to be elucidated. In vitro studies imply repression of apoCIII expression via interaction with a PPRE in the Rev-erb-α promoter, since it has been shown that mice deficient in this protein exhibit increased plasma concentrations of TG and apoCIII [69]. Whether apoCIII affects TG metabolism in vivo is contentious. Some studies showed an effect in normolipidemic subjects [70, 71]. Increases in the number and apo CIII content of VLDL particles also have adverse consequences for other lipoprotein subspecies, contributing to an increase in small, dense LDL particles [72]. The role of fenofibrate in the prevention of atherosclerosis is still disputed with large trials such as the field study[73] failing to demonstrate benefit for primary endpoints although secondary endpoints suggested that benefit and the DAIS study [74], an angiographic study in diabetes, certainly demonstrated significant reduction in the progression of atherosclerotic lesions (Figure 1).

The role of Apo A-IV is of interest as it increases MTP activity and leads to increased lipidation of the chylomicron particle (For review see Black [75]). In newborn swine, intestinal epithelial cells that had overexpressed apo A-IV increased the lipid content of the chylomicron particle [76, 77]. Further studies showed that the mechanism was through upregulation of MTP at the pretranslational level [78]. A meal rich in fat increases Apo A IV synthesis. During lipolysis of the chylomicron particle apo A IV binds to HDL although some circulates free. The function of apo AIV on HDL is not clear, but reduced levels are associated with cardiovascular disease, and transgenic overexpression protects mice fed a high-fat diet from atherosclerosis [79] suggesting that Apo A IV plays an important regulatory

role in fat absorption and storage. Fasting jejunal lipid content was examined in morbidly obese persons some of whom had diabetes [80]. The diabetic subjects had lower triglyceride levels but Apo A IV mRNA was significantly higher with a significant negative correlation between apo IV mRNA expression and jejunal triglyceride. The diabetic patients had higher chylomicron triglycerides and apo B48. In the fructose-fed hamster, a model of insulin resistance, it has been demonstrated that the intestine is not responsive to the insulin-induced downregulation of the apo B48 lipoprotein production found in the chowfed animals [81]. The mechanism appears to be through disturbance in the ERK pathway which involves both insulin signaling and lipoprotein overproduction. These results are in keeping with the studies which have shown that insulin resistance and diabetes are associated with an increase in MTP in the liver and that MTP is negatively regulated by insulin [82–84]. In further studies reported by the Toronto group in 2010 [85] the prechylomicron transport vesicles (PCVs) were characterized, and proteomic profiles were developed. They have reported that MTP, Apo B48, SAR-1, and VAMP-7 were differentially expressed when compared to the chow fed animals. The results the authors suggest have increased our understanding of the assembly and transport of nascent chylomicrons in insulin-resistant states.

The intestinal enterocyte has a short half-life but yet manages to finely control fat absorption so that even the largest fat meal does not pass through unabsorbed. The mechanism involves increase in chylomicron number as shown by increased apo B and increased size as shown by the fat content of the chylomicron. Dai et al. [86] examined the mechanism whereby CaCo 2 cells differentiate into enterocyte cells and secrete chylomicron-like apo B48 particles when incubated with oleic acid, whereas cells that go on to become crypt cells do not have this ability. They found that MTP expression seemed to be a limiting factor for apo B lipoprotein secretion. They found evidence that HNF1, HNF4, and DR1 were critical for differentiation-dependent MTP induction and that repression was induced by NR2F1 and IRE1B. NR2F1 and IRE1B were found more in the crypts than in the villi suggesting the mechanism whereby only the enterocyte has the facility to absorb fat and that MTP is the limiting factor. These experiments add to the excitement of intestinal MTP as a target for treatment of dyslipidaemia in diabetes and other conditions where there is excess of postprandial chylomicrons.

8. Gene Regulation of Chylomicron Metabolism

A high-fat diet is associated with an increase in triglyceride-rich lipoproteins. Hernández-Vallejo et al. [87] investigated the impact of a short-term high-fat diet in mice and showed that apo B, MTP, and Apo A IV were upregulated to handle the increased lipid load. They also showed that there was a suppression of genes associated with fatty acid synthesis, FAS, ACC, SREBP-1c, and a key regulator of lipid biosynthesis was increased and translocated to the nucleus. LXR is considered to be a central player in energy homeostasis as indicated by its putative role in lipogenesis, gluconeogenesis, lipoprotein metabolism, and glucose uptake [88]. SREBP was only partly dependent on LXR. Apo A5 plays a major role in the metabolism of triglyceride-rich lipoproteins in the liver [89]. It is less certain whether apo A-5 plays a part in the chylomicron metabolis. Recently Guardiola et al. [90] have demonstrated gene expression mostly in the duodenum and colon. In vitro studies suggested that the protein may be functional, but this needs further investigation.

Obesity is associated with an increase in inflammatory cytokines such as TNF-a [91]. TNFa infusion has been shown to stimulate the overproduction of intestinal apo B48 as well as hepatically derived apo B 100 particles [92, 93]. TNF a increases MTP, and we found MTP to be increased in animal and human diabetes [26, 27, 48, 49], a condition that is associated with increased TNFa [94]. Recently Qin et al. [94] have shown that TNFa increased CD 36 which is, among other functions, an important fatty acid transporter. Thus a vicious cycle is put in place whereby excessive feeding increases chylomicron production which leads to insulin resistance through deposition of fat which in turn stimulates TNFa and other cytokines which increase insulin resistance which increases chylomicron production.

The delipidated chylomicron particle is cleared by the liver through the LDL receptor-related protein 1, (LDLR-related protein 1) receptor, and LDL B/E receptor. The LDLR is insulin sensitive, and the receptor is downregulated in insulin resistance [95]. Through a series of steps the lipid and cholesterol are repackaged and excreted by the liver as VLDL with apoB100 as the solubilising protein. The pathways in the liver are not dissimilar to those in the intestine, and like chylomicrons in the intestine, the VLDL particle will contain some de novo synthesised cholesterol. The liver like the intestine can regulate, at least to some extent, the amount of cholesterol in the VLDL particle by regulation of excretion of cholesterol through the bile. NPC1L1 plays a part in the liver in the regulation of cholesterol transport. Hepatic nuclear factor-1 (HNF-1) alpha and sterol regulatory element binding protein (SREBP)-2 appear to be important regulators of NPC1L1 in the liver [96]. It has also been shown that they have important binding sites within the human NPC1L1 promoter. The role of NPC1L1 in the liver is probably to divert cholesterol away from excretion in the bile [97]. A recent study in female Chinese women with gall stones has shown reduced NPC1l1 mRNA and protein in the liver and supersaturation of cholesterol in the bile [98]. Ezetimibe has not been shown to increase the risk of gall stones perhaps because the drug has its primary effect in reducing cholesterol absorption. Indeed in the golden Syrian hamster Ezetimibe reduced diet-induced increase in biliary cholesterol [99], and, in gallstone-susceptible mice fed lithogenic diets, Ezetimibe prevented gall stone formation [100]. Inhibition of NPC1L1 by Ezetimibe is associated with an improvement in hepatic steatosis. Jia et al. [101] have recently investigated the mechanism by deleting NPC1L1 in mice and inducing hepatic steatosis with a high fat diet. The knockout mice did not develop steatosis. Hepatic fatty acid synthesis and mRNA for genes regulating lipogenesis were reduced, and the knockout animals did not develop hyperinsulinaemia. Nomura et al. [102] demonstrated in

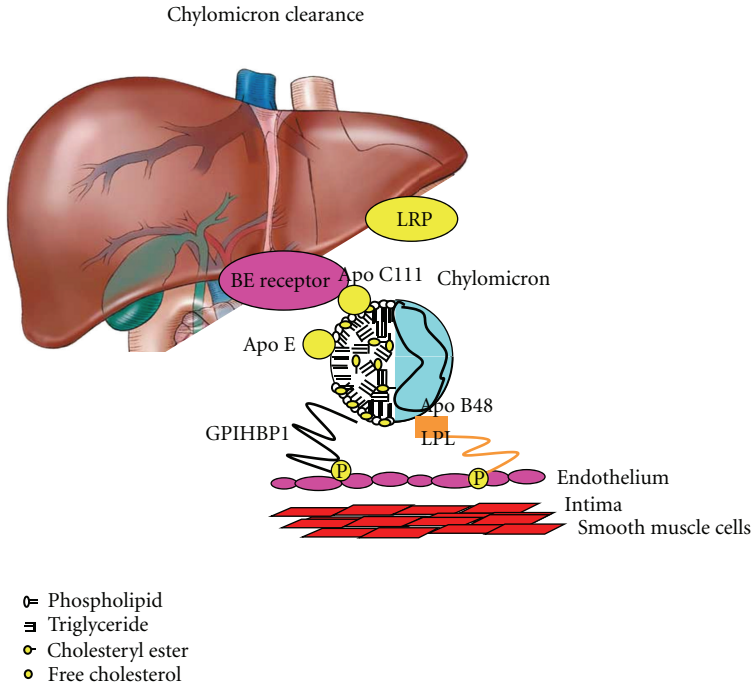

Figure 2: Chylomicron clearance. The chylomicron acquires apo E and apo C111 in the circulation, and most of the triglyceride is hydrolised by lipoprotein lipase (LPL) prior to it being cleared by the low density lipoprotein (LDL) B/E receptor or the LDL receptor-related protein (LRP) receptor in the liver. GPIHB also plays a smaller part in uptake of chylomicron remnants by the liver capillaries.

Zucker rats that Ezetimibe improved hepatic insulin signaling as well as hepatic steatosis in both the liver and in cultured steatotic hepatocytes. The drug recovered insulin-induced Akt activation and reduced gluconeogenic genes. The relevance of this study to humans is not clear as patients with diabetes who are treated with Ezetimibe do not improve blood sugar control Kishimoto et al. [103].

9. Chylomicron Clearance

Delayed clearance of the chylomicron particle is another cause of hypertriglyceridaemia. The increased residence time of the particle potentially increases its atherogenicity and may be an important factor in the development of small dense LDL since large triglyceride-rich particles correlate well with small dense LDL. The delay in clearance of the chylomicron inevitably leads to a delay in clearance of VLDL since the apo BE receptor preferentially takes up the chylomicron particle. The triglyceride-rich lipoproteins may also be cleared in the liver by heparin sulfate proteoglycans HSPGs [104–106]. Among the HSPGs are the transmembrane syndecans, which have been shown to mediate the internalisation of model lipoproteins. Syndecan 1 can mediate binding and uptake of chylomicron remnants by HepG2 liver cells [107]. Stanford et al. [108] have shown this in in vivo genetic studies, using knockdown mice. Williams [104] showed that glycosylphosphatidyl inositol anchored high-density lipoprotein binding protein 1 (GPIHBP1) plays a critical role in the lypolytic processing of chylomicrons. GIHBP1 is located on the luminal face of the capillary endothelium and has been shown to bind both LpL and chylomicrons [109] suggesting that it serves as a platform for lipolysis drawing the chylomicron and LpL into close proximity. The role in atherosclerosis of this protein has still to be explored; however, a missense mutation of GIHBP1 in a young boy with severe chylomicronaemia has been described, and one presumes that other polymorphisms with less severe chylomicronaemia will help to unravel the role in atherosclerosis of this protein. Another cause for hypertriglyceridaemia is a mutation in HSPGs. Lipoprotein lipase (LpL) is stored in the subendothelial compartment awaiting transport. GPIHBp facilitates the transport of LpL from the subendothelium to the luminal side of the vasculature [110]. Bishop et al. [111] have demonstrated a deletion of the HPSG, Collagen 18, which resulted in reduced vascular LpL mass and activity in mice and caused mild hypertriglyceridaemia. Patients with collagen 18 deficiency have Knoblauch syndrome, a rare disorder characterised primarily by ocular defects. These patients have hypertriglyceridaemia (Figure 2) [111].

10. Chylomicron and Atherosclerosis

Whereas cholesterol is tightly regulated with carefully evolved mechanisms to ensure that dietary cholesterol deficiency does not impede the body's need for cholesterol, the triglyceride metabolism is exquisitely regulated so that almost no dietary fat is lost, energy being conserved for later famine. The atherosclerotic plaque is mostly made up of cholesterol, fatty acids, and fibrous tissue. The recognition

that, in conditions of severe hypercholesterolaemia such as familial hypercholesterolaemia and familial combined hyperlipidaemia, life expectancy was severely reduced due to atherosclerosis focused attention on cholesterol as being a major player in the atherosclerotic process. Population studies have confirmed the association. LDL cholesterol is the major cholesterol-containing particle. If cholesterol is measured per particle the LDL particle contains 100-fold more as compared to the chylomicron and the statins, which inhibit cholesterol synthesis and have been demonstrated to lower mostly LDL cholesterol and to reduce atherosclerosis. This has tended to focus attention even more strongly on the LDL particle as being the major player in atherosclerosis to the determent of the chylomicron. The chylomicron cholesterol content may be low, but the half-life is in minutes (rather than around 4 days for the LDL particle), hence the cholesterol carrying power of these particles is enormous and similar to LDL. More than 10 years ago Karpe et al. [112] demonstrated a relationship between apo B48 and carotid atherosclerosis both in normotriglyceridaemic and hypertriglyceridaemic subjects An argument that the chylomicron particle could not be considered as an atherogenic particle because of its size and therefore its inability to enter the subendothelial space is no longer valid since apo B48 has been found in atherosclerotic plaques in both animal and human studies [113, 114]. Proctor and Mamo [113] have demonstrated apo B48 in rabbit atherosclerotic plaque. The authors in elegant studies have also demonstrated that perfusing both chylomicron remnants and LDL in the rabbit aorta resulted in an preferential uptake of apo B48 in the subendothelial space suggesting that the chylomicron does indeed play a predominant role in the delivery of not only cholesterol but also of fatty acids to the plaque. Pal et al. in 2003 examined carotid endarterectomy patients and found apo B48 in the plaque [114]. These findings have been confirmed in humans by other workers [115]. The mechanism whereby the macrophage takes up the chylomicron particle has been extensively investigated. The chylomicron remnant competes for uptake of native LDL through the LDL receptor [116] but an apo B48-specific receptor has also been described [117, 118]. Elsegood et al. [119, 120] have described a 43 Kda macrophage chylomicron remnant binding protein as a candidate for sterol loading of macrophages resulting in the unabated uptake of chylomicron remnants by macrophages. It has been suggested that the chylomicron particle contains too little cholesterol to make it an important transporter of cholesterol to the atherosclerotic plaque but it must be remembered that the chylomicron particle number is massive in the postprandial phase compared to LDL and has a half-life in minutes rather than in days thus transporting over time, in relative terms, a similar amount of cholesterol compared to LDL. There have been as yet no clinical studies that have investigated the relationship between apo B48 and cardiovascular events, but it has been suggested that there is enough evidence to mount such trials [121]. Patients with type 1 diabetes as in type 2 diabetes are at increased risk of atherosclerosis. Mangat et al. [122] demonstrated increased apo B48 both fasting and postprandial in type 1 diabetic patients compared to controls.

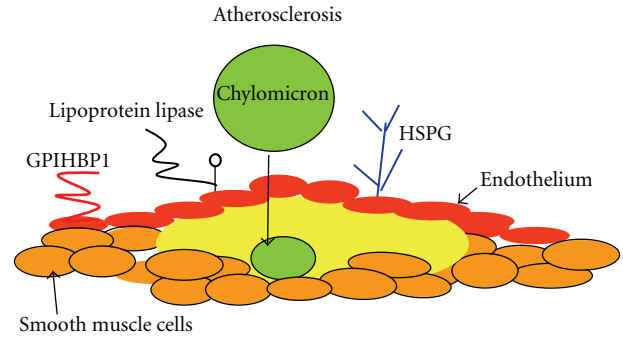

Figure 3: The chylomicron and atherosclerosis. The atherosclerotic plaque is composed of a lipid-rich core containing cholesterol and necrotic tissue and is covered by a fibrous smooth muscle cell cap. Low density lipoprotein (LDL) is the major contributor to plaque cholesterol, but chylomicron remnants are also taken up into the subendothelial space, and because of the rapid turnover of chylomicrons the amount of cholesterol they can deliver to the plaque is not reflected in serum chylomicron cholesterol. Chylomicrons are delipidated by lipoprotein lipase on the artery wall. They are attached to the endothelium by high-density lipoprotein-binding protein 1 (GPIHBP1) and heparin sulphate proteoglycans (HPSG) which facilitate their uptake into the subendothelial space. Chylomicron remnants become trapped in the artery wall and disintegrate to contribute cholesterol to the lipid-rich core.

They also showed that the arterial retention of remnants ex vivo was increased 7-fold in type 1 diabetes relative to controls. The authors also showed that the remnants bound with significant affinity to human biglycan in vitro with a further 2-3-fold increase in binding activity with glycated glycan. The authors suggest that their findings support the hypothesis that impaired remnant metabolism may contribute to accelerated progression of atherosclerosis. We and others have shown increased Apo B48 in type 2 diabetes as compared to controls [123–126]. This abnormality was improved with better control of diabetes [123]. Taskinens group [126] has demonstrated an increase in apo B48 in diabetic patients with CVD as compared to diabetic patients without atherosclerosis. Finally it should be remembered that an increase in chylomicron cholesterol leads to and correlates with both VLDL cholesterol and LDL cholesterol, and large triglyceride-rich lipoproteins are associated with the atherogenic small dense LDL and low HDL. Both of these findings demonstrate the importance of the chylomicron in generating an atherogenic lipoprotein profile (Figure 3).

In conclusion there is clear evidence that chylomicron metabolism is abnormal in diabetes, a condition which is associated with a heavy burden of atherosclerosis. There is good evidence to implicate the chylomicron directly in the atherosclerotic pathological process, and there is good evidence that abnormal chylomicron metabolism is associated with an atherogenic LDL profile. Measures to decrease chylomicron formation should decrease atherosclerotic burden, and the results of an intestinal MTP inhibitor are therefore awaited with great interest. Increasing chylomicron turnover may not prove useful since accelerated metabolism of the particle may just lead to increased deposition in the

plaque and increased VLDL and an increase in small dense LDL. In both diabetic and nondiabetic subjects it seems wise to restrict chylomicron formation through strict diet and in diabetes; meticulous control of blood sugar will also improve chylomicronaemia. It is time to focus our attention on the chylomicron as an important player in the atherosclerotic process. The experimental animal studies and small human studies make it apparent that the time is right for large prospective clinical trials to evaluate the dangers of postprandial chylomicronaemia. With the advent of the specific intestinal inhibitors of MTP, it is likely that these studies will soon be undertaken.

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Research Article

Lipofundin-Induced Hyperlipidemia Promotes Oxidative Stress and Atherosclerotic Lesions in New Zealand White Rabbits

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Atherosclerosis represents a major cause of death in the world. It is known that Lipofundin 20% induces atherosclerotic lesions in rabbits, but its effects on serum lipids behaviour and redox environment have not been addressed. In this study, New Zealand rabbits were treated with 2 mL/kg of Lipofundin for 8 days. Then, redox biomarkers and serum lipids were determined spectrophotometrically. On the other hand, the development of atherosclerotic lesions was confirmed by eosin/hematoxylin staining and electron microscopy. At the end of the experiment, total cholesterol, triglycerides, cholesterol-LDL, and cholesterol-HDL levels were significantly increased. Also, a high index of biomolecules damage, a disruption of both enzymatic and nonenzymatic defenses, and a reduction of nitric oxide were observed. Our data demonstrated that Lipofundin 20% induces hyperlipidemia, which promotes an oxidative stress state. Due to the importance of these phenomena as risk factors for atherogenesis, we suggest that Lipofundin induces atherosclerosis mainly through these mechanisms.

1. Introduction

Atherosclerosis is a chronic vascular disease and a leading cause of death in the western world. It is well established that hyperlipidemia and oxidative stress (OS) are major contributors to atherogenic development [1]. The retention of low-density lipoproteins (LDL) in the arterial wall [2] and their oxidation by reactive oxygen species (ROS) initiates a complex series of biochemical and inflammatory reactions [3, 4]. Oxidized LDL (ox-LDL) are internalized by macrophages through the scavenger receptors, leading to foam cell formation [5]. Furthermore, oxidized cholesterol products present in blood and in arterial plaques increase cholesterol biosynthesis, affect plasma membrane structure, cell proliferation, and cell death, and promotes atherosclerosis development [6].

The rabbit is one of the most widely used animal models in atherosclerosis research. One strategy to induce atherosclerotic lesions in these animals is through an intravenous administration of Lipofundin 20%, a lipid-rich emulsion used in parenteral nutrition, which produces aortic lesions, characterized by subendothelial lipid accumulation, intimal thickening, and a distortion of vascular tissue architecture [7, 8]. The impact of Lipofundin 20% administration on lipid levels and redox environment in New Zealand white (NZW) rabbits had not been studied. In the present work, we demonstrated that Lipofundin 20% induces a hyperlipemic

state and a systemic/aortic oxidative stress, which can lead to atherosclerotic lesions development.

2. Materials and Methods

2.1. Animals. Standard NZW male rabbits, weighing 2.0–2.5 kg and 12 weeks old, were obtained from CENPALAB (Bejucal, Havana, Cuba). Rabbits were housed under conventional conditions exposed to light-dark cycle of 12 h with free access to water and food. Animal studies were performed with the approval of Pharmacy and Food Sciences College Institutional Animal Ethical Committee. All procedures were performed in accordance with the guidelines stipulated by the Institutional Animal Care Committee and the European Union Guidelines for animal experimentation.

2.2. Lipofundin Composition. Lipofundin MCT/LCT 20% (Braun Melsungen AG, Melsungen, Germany) is a lipid emulsion containing soya oil 100 g, medium-chain triglycerides 100 g, glycerol 25 g, egg lecithin 12 g, α-tocopherol 170 ± 40 mg, and sodium oleate/water for injection in sufficient quantity to 1000 mL.

2.3. Experimental Design. Two groups of 10 rabbits were used in the study. The first group received an intravenous injection of phosphate-buffered saline (PBS), pH 7,4 (control group), and the second one received a slow intravenous injection of 2 mL/kg of Lipofundin MCT/LCT 20%, as an infusion during 1-2 min [7, 8]. This procedure was repeated daily during a period of 8 days. On day 9, the animals were anesthetized with ketamine hydrochloride (5 mg/kg i.m.) and euthanized with an overdose of sodium pentobarbital (90 mg/kg, i.v.). (Abbott Laboratories, Mexico SA de CV, Mexico), and the vascular system was perfused with NaCl 0.9% solution at 4°C. Then, aortas were excised from the aortic arch to abdominal segment, and adventitial fat was removed. Aortic arches were used for histopathology and redox evaluations due to the preferential development of Lipofundin 20%-induced atherosclerotic lesions in this segment [8]. For each evaluation, the samples of five animals per group were used.

2.4. Serum Sample Collection. Blood samples (3 mL) were obtained on day 0 (before Lipofundin administration) and on day 9 (at the end of the study), for biochemical analyses. Blood was withdrawn from the rabbit's marginal ear vein. These samples were immediately centrifuged at 2500 g, at 4°C for 10 min. The serum was collected and aliquots were stored at −80°C until analysis.

2.5. Aortic Homogenate Preparation. Aortic arches were placed in ice-cold 0.1 mol/L Tris-HCl buffer, pH 7,6 containing 1.0 mmol/L EDTA and 0.2 mmol/L butylated hydroxytoluene (buffer A) and macerated before homogenization in a tissue homogenizer (Edmund Bühler LBMA, Germany). Homogenized tissue was then centrifuged at 4500 g for 20 min at 4°C, and the supernatants were collected and stored at −80°C until redox biomarkers determinations.

2.6. Histopathology

2.6.1. Eosin-Hematoxylin Staining. Aortic arches were rinsed in PBS, pH 7,4, transversally cut, and fixed in 10% formaldehyde solution. Samples were then embedded in paraffin. Five-micrometer tissue sections were cut, air-dried on glass slides, deparaffinized, and rehydrated. Finally, tissue sections were stained with eosin and hematoxylin (HE) under standard procedures. The sections were analyzed in an optic microscope Olympus BX51.

2.7. Ultrastructural Analysis

2.7.1. Electron Transmission Microscopy. For transmission electron microscopy (TEM), samples from rabbit aortic arch were fixed for 1 h at 4°C in 3.2% glutaraldehyde (Agar Scientific, UK), 0.1 M phosphate buffer (pH 7,4) and postfixed in 1% OsO_4 for 1 h. After graded ethanol dehydration, samples were embedded in Spurr low-viscosity epoxy resin for 24 h at 37°C. Ultrathin sections were cut into 400–500 Å thick slice with an ultramicrotome (NOVA, LKB), counterstained with uranyl acetate and lead citrate, and analyzed in a TEM (JEOL JEEM-2000EX, JEOL, Japan).

2.7.2. Serum Lipid Assay. Serum total cholesterol, triglycerides, LDLc, and HDLc were determined using commercial enzymatic kits (Randox, Crumlin, UK).

2.7.3. Redox Biomarkers Determinations. All biochemical parameters were determined by spectrophotometric methods using a Pharmacia 1000 Spectrophotometer (Pharmacia LKB, Uppsala, Sweden). Total proteins levels were determined using the method described by Bradford [9] with bovine serum albumin as standard. SOD activity was determined by using RANSOD kit (catalogue no. SD 125, Randox Labs, Crumlin, UK), where xanthine and xanthine oxidase were used to generate superoxide anion radicals ($O_2^{\bullet -}$), which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to form a red formazan dye. SOD activity was measured by the inhibition degree of this reaction. Catalase (CAT) activity was determined by following the decomposition of hydrogen peroxide (H_2O_2) at 240 nm at 10 s intervals during 1 min [10].

After precipitation of thiol proteins, the reduced glutathione (GSH) levels were measured according to the method of Sedlak and Lindsay [11] with Ellman's reagent (5,5′dithiobis-2-nitrobenzoic acid) (Sigma St. Louis, MO, USA), and the absorbance was measured at 412 nm. Purified GSH (Sigma St. Louis, MO, USA) was used to generate standard curves.

The advanced oxidation protein products (AOPPs) were measured as described previously [12]. Briefly, samples in PBS (1 mL) were treated with 50 μL of potassium iodide 1.16 M followed by the addition of 100 μL of acetic acid. The absorbance was immediately read at 340 nm. AOPP concentration was expressed as μM of chloramines-T.

Concentration of malondialdehyde (MDA) was determined using the LPO-586 kit obtained from Calbiochem (La Jolla, CA, USA). In the assay, the production of a stable

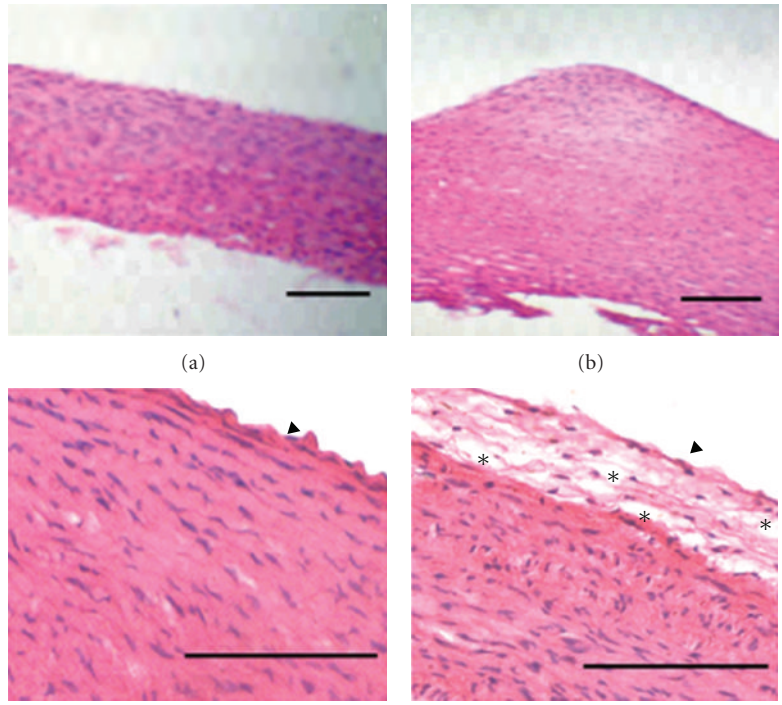

Figure 1: Histopathological analysis of rabbit's aortic tree. Eosin/hematoxylin staining reveals a normal morphology of aortas in control animals (a) and (c), while aortas of Lipofundin group show an intimal thickening, characterized by a vascular tissue architecture distortion and large extracellular spaces, probably filled with lipids (asterisks) (b) and (d). Arrow head: endothelial cells. Magnification 10× (a) and (b) and 40× (c) and (d). Scale bar, 20 μm.

chromophore after 40 min of incubation at 45°C was measured at 586 nm. For standards, freshly prepared solutions of malondialdehyde bis (dimethyl acetal) (Sigma St. Louis, MO, USA) were employed and assayed under identical conditions [13, 14].

In order to determine susceptibility to lipid peroxidation and total reactive antioxidant power (TRAP), the samples were incubated with a solution of copper sulphate (final concentration 2 mM) at 37°C for 24 h. The peroxidation potential (PP) was calculated by subtracting the MDA levels before the induction of lipid peroxidation from the one obtained at 24 h [15].

Nitrites (NO_2^-) level, as a surrogate marker of nitric oxide ($NO^\bullet$), were determined converting nitrates to nitrites using nitrate reductase (Boehringer Mannheim Italy SpA, Milan, Italy). Then, Griess reagent (1% sulphanilamide, 0.1% N-(1-Naphthyl)-ethylenediamine dihydrochloride in 0.25% phosphoric acid) was added [16]. Samples were incubated at room temperature for 10 min, and absorbance was measured at 540 nm.

2.8. Statistical Analysis. Statistical analysis was performed using the SPSS program for Windows (version 11.5, SPSS Inc). Bartlett's Box-test was used to test the homogeneity of variance. Differences between groups were determined by student's *t*-test (two-tailed). Data were expressed as the mean ± standard deviation (SD). A P value of < 0.05 was considered statistically significant.

3. Results

3.1. Histopathology. The HE staining of aortic arch sections from control rabbits showed neither intimal thickening nor distortion in the vascular tissue architecture (Figures 1(a) and 1(c)). In contrast, aortic sections from those animals who received intravenously 2 mL/kg of Lipofundin 20% during 8 days showed a thickening of the intima with apparent lipid accumulation and distortion of tissue architecture (Figures 1(b) and 1(d)).

Nonrelevant disease or abnormalities in other organs were detected by macroscopic and microscopic examination.

3.2. Ultrastructural Analysis. On the other hand, the ultrastructural analysis confirmed the results observed by light microscopy. In the animals treated with Lipofundin was observed an endothelial damage characterized by a loss of endothelium integrity and the presence of abundant foam cells and myofibroblasts in the intima and media layers. Also, we observed a high extracellular lipid accumulation and collagen fibers deposition (Figures 2(c), 2(d), 2(e), 2(f)). No alterations in the aortic artery wall of control rabbits were observed (Figures 2(a) and 2(b)).

3.3. Serum Lipids. Serum total cholesterol, triglycerides, LDLc, and HDLc levels showed a significant increase ($P < 0.05$) in those animals who were treated during 8 days with the lipid-rich emulsion Lipofundin, while no significant

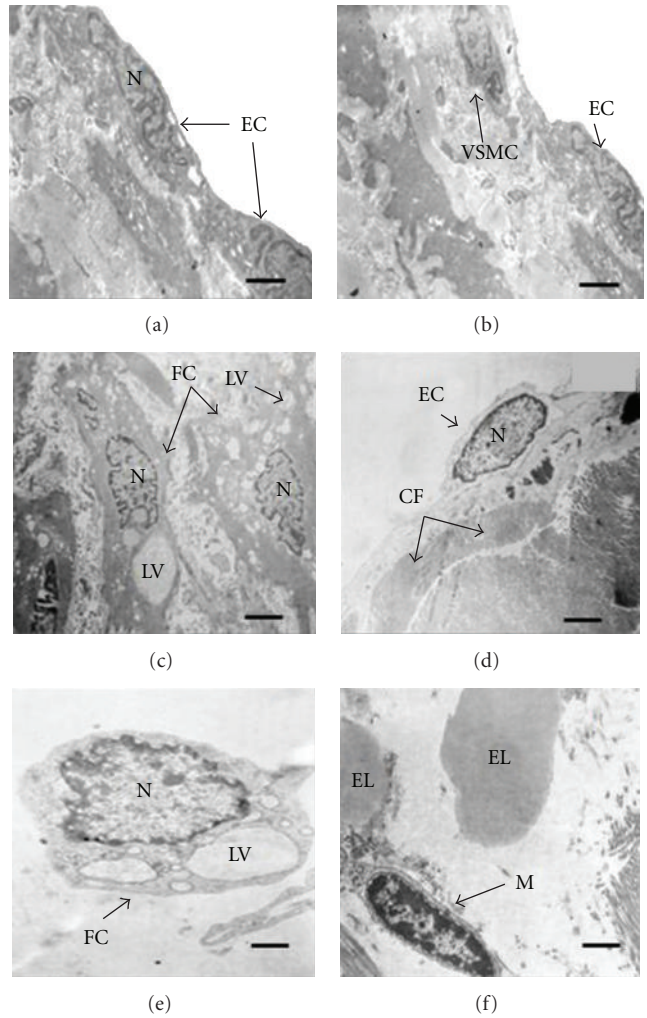

Figure 2: Ultrastructural analysis. Panels (a) and (b) correspond to animals of control group, while panels (b), (c), (d), and (e) show the effects of Lipofundin administration on atherosclerotic lesion formation. EC: endothelial cells, EL: extracellular lipids, VSMC: vascular smooth muscle cells, N: nucleus, FC: foam cells, LV: lipid vacuolization, CF: collagen fibers, M: myofibroblasts. Scale bar 1 μm (a, b, c, d), 500 nm (e, f).

changes in serum lipids were observed in the control rabbits throughout the study (Table 1).

3.4. Redox Biomarkers. Table 2 shows the behavior of serum and aortic redox parameters in both groups. The biomolecules damages markers were significantly ($P < 0.05$) modified after 8 days of Lipofundin administration compared to nontreated group. At the end of the experimental period, the MDA levels, one of the end-products of lipid peroxidation, were higher in Lipofundin-treated animals compared with controls. Besides, Lipofundin treatment also caused a rise of AOPP levels in comparison with control group. The activity of both antioxidant enzymes SOD and CAT were significantly higher ($P < 0.05$) in Lipofundin group at the end of the experiment compared to control rabbits. The NO_2^- levels and GSH concentration decreased significantly after 8 days of Lipofundin treatment in comparison to those of untreated animals ($P < 0.05$). Finally, the susceptibility to lipid peroxidation was higher in those animals who received Lipofundin. After 8 days, in these animals was observed a significant increase of PP ($P < 0.05$), compared to the one calculated in controls.

Table 1: Effects of Lipofundin on serum lipid profile. Values represent the mean ± standard deviation. Asterisks represent statistical differences ($P < 0.05$).

	Control	Lipofundin
TC, mmol/L	1.78 ± 0.06	3.10 ± 0.13*
TG, mmol/L	1.51 ± 0.03	2.73 ± 0.07*
HDLc, mmol/L	0.76 ± 0.04	1.20 ± 0.04*
LDLc, mmol/L	0.18 ± 0.01	0.83 ± 0.03*

4. Discussion

The histopathological analyses of the aortic sections from rabbits treated with Lipofundin 20% demonstrated the

Table 2: Effects of Lipofundin on redox biomarkers. Values represent the mean ± standard deviation. Asterisks represent statistical differences ($P < 0.05$). The concentration of aortic parameters is expressed per milligrams of total proteins (Pr).

	Control	Lipofundin
Systemic redox biomarkers		
MDA, μM	2.69 ± 0.07	6.24 ± 0.28*
AOPP, μM of chloramines	11.50 ± 0.73	16.22 ± 0.47*
PP, μM of MDA	4.63 ± 0.18	9.13 ± 0.34*
CAT, U/L/min	351.13 ± 19.03	477.50 ± 30.46*
SOD, U/mL/min	22.03 ± 26.44	32.00 ± 1.60*
NO_2, μM	179.18 ± 11.44	134.33 ± 5.09*
GSH, μM	309.03 ± 26.44	191.21 ± 8.26*
Aortic redox biomarkers		
MDA, μM/mgPr	18.49 ± 2.04	27.42 ± 2.55*
AOPP, μM of chloramines/mgPr	12.45 ± 1.21	24.25 ± 1.86*
PP, μM of MDA/mgPr	13.81 ± 1.83	25.26 ± 2.29*
CAT, U/L/min/mgPr	1023.60 ± 26.89	1609.68 ± 84.37*
SOD, U/mL/min/mgPr	62.37 ± 3.93	105.39 ± 9.82*
NO_2, μM/mgPr	95.29 ± 2.54	43.96 ± 6.03*
GSH, μM/mgPr	166.70 ± 12.82	71.59 ± 10.89*

capacity of Lipofundin to induce atherosclerotic lesions. As described above, an intimal thickening and a distortion of tissue architecture was observed by EH staining. Electron microscopy confirmed the presence of foam cells, extracellular lipid accumulation, collagen fibers deposition, vascular smooth muscle cells (VSMC) migration, the presence of myofibroblasts, and also the loss of endothelium integrity. These events, induced by Lipofundin 20%, contribute with the development and progression of atherosclerosis.

At the end of the experiment, we observed high serum levels of triglycerides, total cholesterol, LDLc, and HDLc in the animals treated with Lipofundin 20% in comparison to control rabbits. Indeed, there is a causal relationship between the elevated plasma lipids and the development of atherosclerotic lesions [17–19].

Lipofundin 20%-induced hyperlipidemia could be associated with the high content of triglycerides in this emulsion. High levels of exogenous triglycerides promote ApoB100 and cholesterol synthesis and eventually the assembly of very low-density lipoproteins (VLDL) [20]. In fact, Lipofundin 10% caused a 60% increase in total serum cholesterol after parenteral administration in a human study [21].

In addition, there is a mutual exchange of lipids and apolipoproteins between serum lipoproteins and the infused triglyceride/phospholipid particles [22]. The increase of HDLc may be determined by a physiological response against the elevated LDLc levels. It is known that HDL protect from atherosclerotic development. However, based on recent animal and epidemiological studies, it appears that in addition to quantity [23] other properties of HDL, such as antioxidant and anti-inflammatory power, are necessary for atheroprotection [24, 25].

In this study, we demonstrated that Lipofundin-induced hyperlipidemia was associated with a systemic and aortic OS. Strong evidences for the involvement of free radicals production in the onset of hyperlipidemia have been reported previously [26]. Chronic generation and sustained high toxic levels of ROS are associated with several pathological conditions including cardiovascular diseases such as atherosclerosis [27]. During atherosclerotic lesions development, cellular damages take place through mechanisms involving lipid peroxidation and oxidative modifications of proteins [28]. On the other hand, a disruption of antioxidant enzymes activity and a drastic reduction of nonenzymatic defenses are also observed during atherogenesis [29]. High levels of MDA in the sera and aortic tissue from rabbits bearing atherosclerotic lesions, compared with those from control group, suggest the role of LPO in the loss of redox cellular status in the former animals which were under atherogenic stimuli caused by Lipofundin treatment. MDA levels have been considered not only an indicator of OS, but also as a biochemical marker of atherogenesis [30, 31].

Oxidative modifications of proteins have been also implicated in atherosclerosis [32]. Through AOPP determination, we measured the chlorinated proteins levels, caused by myeloperoxidase-derived hypochlorous acid (HOCl). It has been shown that HOCl-modified proteins are present in atherosclerotic lesions and predict the progression of the disease [5]. The high levels of AOPP in those animals that received the lipid emulsion suggest an active role of macrophages infiltration and inflammatory process in the development of atherosclerotic lesions in the present animal model.

Antioxidant defenses, as expression of the balance between generation and inactivation of oxidized metabolites, represent a useful tool to examine the redox status [33, 34]. In our study, the higher activity of extracellular SOD, detected in the animals treated with Lipofundin, could be associated with an increase in $O_2^{\bullet -}$ generation, typically produced by foam cells and macrophages at atherosclerotic lesion sites [35]. Also, in atherogenic process, there is an increase in vascular NADPH oxidase activity, the main source of $O_2^{\bullet -}$ in the vasculature [36].

CAT is another antioxidant enzyme present in the vasculature, which plays an important role on redox environment maintenance [37]. In our study, we found a high activity of the enzyme in animals treated with Lipofundin. During the beginning and development of atherogenic lesions, the enzyme gene expression increases and in this way contributes to retard the disease progression [38, 39]. Also, it has been shown that in early steps of atherogenesis CAT activity is incremented in response to oxidant stimulus mediated by ox-LDL and ROS such as H_2O_2 and lipoperoxides [40].

During atherogenesis, the reactive molecules that are produced have the potential to deplete the surrounding cells of their GSH levels, affecting their antioxidant defenses and detoxification pathways [41]. Our results showed a significant depletion of serum and aortic GSH levels in the animals treated with the lipid emulsion compared to the control rabbits. This fact could be associated with the Lipofundin-mediated ROS generation and with the

high concentration of biomolecules damages detected in Lipofundin-treated animals.

Finally, we evaluated the behaviour of NO_2^- levels, as a marker of $NO^\bullet$ bioavailability. $NO^\bullet$ is a vasoactive molecule which has an important role in vascular homeostasis maintenance [42]. The decrease of $NO^\bullet$ bioavailability is considered an important indicator of vascular endothelial dysfunction contributing to atherosclerosis development [43]. Our experimental results showed a reduced bioavailability of $NO^\bullet$ in Lipofundin-treated rabbits compared with controls. This deleterious effect for vascular function may contribute with the Lipofundin-induced atherogenic development.

5. Conclusions

In summary, the present study demonstrated that Lipofundin 20% induces hyperlipidemia, thereby promoting a systemic and aortic OS and also contributing with atherosclerotic lesions formation in NZW rabbits. This work shows novel evidences of Lipofundin-induced oxidative damages on lipids and proteins, the impairment of antioxidant status, and the reduction of nitric oxide levels. These results reinforce the attractive characteristics of Lipofundin to be used as an inductor of experimental atherosclerosis in rabbits. The reduction of experimental time and the associated costs, compared with other established models, is in our opinion the main advantage of this animal model of atherosclerosis.

Conflict of Interests

There are not conflict of interests.

Acknowledgments

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Research Article

The Oxidative State of Chylomicron Remnants Influences Their Modulation of Human Monocyte Activation

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Chylomicron remnants (CMRs) contribute directly to human monocyte activation *in vitro*, by increasing reactive oxygen species (ROS) production and cell migration. In this study, the effects of the oxidative state of CMR on the degree of monocyte activation was investigated. CMR-like particles (CRLPs) were prepared in three different oxidative states, normal (CRLPs), protected from oxidation by incorporation of the antioxidant, probucol (pCRLPs), or oxidised with $CuSO_4$ (oxCRLPs). Lipid accumulation and ROS production were significantly increased in primary human monocytes incubated with CRLPs, whilst secretion on monocyte chemoattractant protein-1 was reduced, but oxCRLPs had no additional effect. In contrast, pCRLPs were taken up by monocytes to a lesser extent and had no significant effect on ROS or MCP-1 secretion. These studies suggest that the oxidative state of CMRs modulates their stimulation of the activation of peripheral blood human monocytes and that dietary antioxidants may provide some protection against these atherogenic effects.

1. Introduction

It is now acknowledged that cardiovascular disease (CVD) is the largest killer in western countries [1]. Atherosclerosis is major cause of CVD, and it has become clear in the past decades that it is a chronic inflammatory disease [2]. Activation of monocytes is an early event in atherogenesis, triggering their adhesion to the endothelium, migration into the arterial intima, and differentiation into macrophages [3–5]. The role of low-density lipoprotein (LDL) in early atherogenesis is also well understood, it accumulates in the developing neointima where it is taken up by macrophages to form foam cells, leading to fatty streak formation, and these effects are greatly enhanced after oxidation of the particles. Oxidized LDL (oxLDL) also stimulates secretion of proinflammatory cytokines, chemokines, and other factors by macrophages, which exacerbates endothelial dysfunction and drives lesion progression [6]. In recent years, however, evidence has accumulated to indicate that lipoproteins of dietary origin are also atherogenic [7, 8].

Lipids from the diet are absorbed in the gut and secreted into lymph in large, triacylglycerol- (TG-) rich lipoproteins called chylomicrons. They pass through the thoracic duct and into the blood where they undergo rapid lipolysis, losing some of their TG to form smaller chylomicron remnants (CMRs), which then deliver the remaining TG, cholesterol, and other lipids to the liver. CMRs have been shown to enter the artery wall as efficiently as LDL and to be retained in the intima [9–11], and particles resembling CMRs have been isolated from the neointima of human atherosclerotic plaques and in animal models of atherosclerosis [12, 13]. Moreover, delayed clearance of CMRs from the blood correlates with the development of atherosclerotic lesions and is associated with consumption of western diets, obesity, and type 2 diabetes [14].

We and others have shown previously that CMRs are taken up and induce foam cell formation in human monocyte-derived macrophages (HMDMs) [15, 16], in macrophages derived from the human monocyte cell line THP-1 [16–18], and in the murine macrophage cell line J774 [19] and that this leads to modulation of the expression of genes involved in lipid metabolism [20]. Furthermore, we have also found that these lipoproteins influence the secretion of proinflammatory chemokines and cytokines by HMDM and THP-1 macrophages via regulation at the transcriptional level [21, 22].

Unlike LDL, CMRs are able to induce their effects on macrophage foam cell formation and on proinflammatory cytokine secretion without prior oxidation [16–22]. However, our earlier work has demonstrated that, in striking contrast to the effects of LDL oxidation, the oxidative state of CMRs is inversely related to their ability to induce foam cell formation. Thus, when CMRs are protected from oxidation by incorporation of antioxidants into the particles, lipid accumulation in macrophages is increased [17, 23], while oxidation of CMRs inhibits their ability to induce foam cell formation [18, 24]. Our studies have also shown that the oxidative state of CMRs plays an important role in their effects on proinflammatory cytokine secretion by macrophages [22].

Since monocytes are the precursors of macrophage foam cells, the processes which activate them and cause their recruitment to the artery wall are crucial early events in atherogenesis. Activation of monocytes during inflammation involves increased secretion of chemokines and cytokines and other vasoactive mediators together with the production of reactive oxygen species (ROS) [25–27], and recent work has suggested that CMRs may influence some of these processes. Castro Cabezas and colleagues have reported that expression of adhesion molecules is upregulated in leukocytes after a fat meal [28, 29], and studies in our laboratory have shown that CMRs are taken up by both primary human monocytes and THP-1 monocytes, causing ROS production, modulation of chemokine and cytokine secretion, and altered chemotaxis [30]. The importance of the oxidative state of CMR in their effects on monocyte activation, however, are not known.

Oxidation of CMR by the lipoxygenase and myeloperoxidase enzymes which are known to oxidize LDL is likely to occur when the particles enter and are retained in the artery wall. In addition, oxidized lipids resulting from the consumption of fat cooked at high temperatures as well as dietary lipophilic antioxidants are carried in the blood in CMR [31, 32]. It is important, therefore, to establish how the oxidative state of the particles may influence their effects on the early stages of atherogenesis. The aim of this study was to determine whether the oxidative state of CMRs influences their uptake by monocytes and subsequent proinflammatory pathways, using primary human monocytes and model chylomicron remnant-like particles (CRLPs).

2. Materials and Methods

All chemicals and tissue culture reagents were from Sigma (Poole, Dorset, UK) unless otherwise stated. Tissue culture plastics from Falcon Discovery Labware range (Fisher Scientific, UK) were used.

2.1. Preparation of CRLPs. CRLPs were prepared as described previously [16]. Briefly, a lipid mixture containing 70% trilinolein, 2% cholesterol, 3% cholesteryl ester, and 25% phospholipids in 0.9% NaCl (w/v) in Tricine Buffer (20 mM, pH 7.4) was sonicated in 22–24 μm for 20 min at 56°C, followed by ultracentrifugation on a stepwise density gradient at 17,000 ×g. To bind ApoE, lipid particles were collected from the top layer after ultracentrifugation and incubated with the d 1.063–1.21 g/mL fraction of human plasma (National Blood Transfusion Service, North London Centre, UK) which had previously been dialysed (18 h, 4°C). ApoE-containing-CRLPs were then isolated by two further ultracentrifugation steps in d 1.006 g/mL at 120,000 ×g, 12 h, 4°C, followed by 202,000 ×g, 4 h, 4°C, and stored at 4°C under argon until required. All preparations were used within one week. To prepare probucol-containing CRLPs (pCRLPs), 1 mg probucol was added to lipid mixture prior to sonication. CRLPs were oxidized (oxCRLPs) by incubation with $CuSO_4$ (20 μM) with shaking for 5 h at 37°C followed by dialysis to remove the $CuSO_4$ (0.9% NaCl, 24 h, 4°C). Control preparations, obtained by a similar procedure to that described for CRLPs, but in the absence of the lipid particles, were included in all experiments to control for possible contamination factors originating from plasma. Data obtained from monocytes incubated with control preparations were similar to those derived from cells incubated in medium alone.

2.2. Isolation of Human Peripheral Blood Monocytes. With approval from the East London Research Ethics Committee, blood was taken by venepuncture from healthy volunteers into 15% EDTA tubes. Monocytes were isolated by negative selection using RosetteSep according to the manufacturer's instructions (StemCell Technologies, London, UK). Cells were resuspended in RPMI containing 2 mM L-glutamine, 10,000 units/mL Penicillin, 10 mg/mL streptomycin, and 10% (v/v) fetal bovine serum (PAA, Somerset, UK). Monocyte preparations were routinely stained with anti-CD14 antibody (Becton Dickinson, Oxford, UK) followed by flow cytometric analysis to verify purity.

2.3. Oil Red O Staining. 1×10^6 monocytes were incubated with CRLPs (15 μg or 30 μg cholesterol/mL) (or a similar volume of control preparation) and incubated at 37°C for 24 h. Cells were adhered to microscope slides by cytospin (Shandon, ThermoFisher Basingstoke, UK) and stained with Oil Red O as described previously [16]. Images were captured using a Leica upright DM4000B brightfield microscope (Leica Microsystems GmbH; Wetzlar, Germany) and the extent of staining analyzed using Volocity (Perkin Elmer, Beaconsfield, UK).

2.4. Measurement of Reactive Oxygen Species (ROS). Monocytes were loaded with dihydrorhodamine-1, 2, 3 (final concentration 100 μM) for 10 min at room temperature and

seeded onto white opaque 96-well tissue culture plates (2.5×10^4 labelled monocytes/well). CRLPs, oxCRLPs or pCRLPs containing 7.5 μg/mL, 15 μg/mL, or 30 μg/mL cholesterol or a similar volume of control preparation were added, and plates were incubated at 37°C for up to 2 h in 5% CO_2. Fluorescence was measured using a Wallac1410 fluorescent microtitre plate reader (Perkin Elmer, Beaconsfield, UK) at 0, 5, 10, 15, 30, and 60 min.

2.5. Measurement of Chemokine Secretion. Monocytes were seeded at 5×10^5 cells/well in 24-well tissue culture plates, CRLPs, oxCRLPs or pCRLPs containing 15 μg/mL, 30 μg/mL cholesterol or a similar volume of control preparation were added and plates were incubated at 37°C/5% CO_2 for 24 h. Cells were pelleted and the supernatants collected, snap frozen, and stored at −80°C until analysis of MCP-1 secretion using ELISA Duoset assay kits according to the manufacturer's instructions (R&D Systems, Oxford, UK).

2.6. Other Analytical Methods. The total cholesterol and triacylglycerol (TG) content of CRLPs was determined by enzymatic analyses (Thermo Fisher Scientific, Waltham, UK). The level of thiobarbituric acid-reacting substances (TBARS) [33] in the CRLP preparations was used to determine the extent of oxidation of the particles.

2.7. Statistical Analysis. Statistical analysis was carried out using Prism (Graphpad, USA). One-way ANOVA followed by Bonferroni's comparison test was used as appropriate. To analyse differences in ROS production, area under curves (AUCs) were calculated with Prism and compared by Student's t-test. $P < 0.05$ was considered statistically significant. All experiments were repeated with at least three separate monocyte isolates and two separate CRLP preparations.

3. Results and Discussion

3.1. Results

3.1.1. Characteristics of CRLPs. The lipid and TBARS content of CRLPs, oxCRLPs, and pCRLPs is shown in Table 1. There were no significant differences in the TG and total cholesterol concentration of the three CRLP types and the TG : TC molar ratio. More importantly, the TG : TC ratio was not significantly different among the three CRLP types. Significant differences in the TBARS and lipid hydroperoxide content of the CRLPs in different oxidative states were apparent, with the values for oxCRLPs being significantly raised and those for pCRLPs significantly decreased as compared to those for CRLPs.

3.1.2. Lipid Accumulation in Primary Human Monocytes after Incubation with CRLP, pCRLP, or oxCRLP. After 24 h incubation, Oil Red O staining for lipid was increased in monocytes coincubated with CRLPs or oxCRLPs, at a concentration of 15 μg/mL cholesterol (Figure 1(a)), and quantitative analysis of the staining density showed that the changes were highly significant in comparison to control cells (Figure 1(b)). Uptake also appeared to be increased after incubation with the higher concentration of CRLP or oxCRLP (30 μg cholesterol/mL); however, in this case the changes did not reach statistical significance. There were no significant differences in staining density between the 15 and 30 μg cholesterol/mL concentrations for any of the CRLP types. In contrast, after coincubation of monocytes with pCRLPs there was no visible increase in lipid accumulation above cultures treated with control preparations, and this was confirmed by staining density analysis (Figure 1(b)).

Table 1: Lipids and TBARS content of CRLP.

Parameter	CRLPs	oxCRLPs	pCRLPs
TG (μmol/mL)	9.44 ± 2.33	6.01 ± 1.31	7.82 ± 1.50
TC (μmol/mL)	0.98 ± 0.24	0.61 ± 0.18	1.03 ± 0.21
TG : TC (molar ratio)	10.1 ± 1.6	12.7 ± 3.1	7.5 ± 0.9
TBARS (nmol MDA/μmol TG)	0.78 ± 0.05	4.00 ± 0.90**	0.39 ± 0.15**

CRLPs, oxCRLPs or pCRLPs were prepared as described in Section 2 and the TG, total cholesterol (TC) and TBARS content was measured. Data are the mean 4 preparations. Significance limits; ** $P < 0.01$ versus CRLP.

3.1.3. Reactive Oxygen Species Generation after Incubation of Primary Human Monocytes with CRLP. To determine the effects of CRLP in different oxidative states on ROS accumulation in monocytes (Figure 2), fluorescence was measured at time points between 0 and 60 min after the addition of CRLPs, oxCRLPs, or pCRLPs to the medium (Figures 2(a)–2(c)) and the area under curve (AUC) was calculated for each experiment (Figures 2(d) and 2(e)). Addition of CRLPs or oxCRLPs led to higher ROS production as assessed by the AUC when compared to incubation with the control preparation (Figures 2(e) and 2(f)), and the changes were significant at concentrations of 15 and 30 μg cholesterol/mL (Figures 2(e) and 2(f)). After incubation with pCRLP; however, there was no increase in ROS generation in comparison to control cells at any concentration of cholesterol (Figures 2(e) and 2(f)).

3.1.4. MCP-1 Production by Primary Human Monocytes after Incubation with CRLP. After incubation of monocytes with CRLP or oxCRLP (15 or 30 μg cholesterol/mL) there was a decrease in MCP-1 secretion compared to control cells (Figure 3), and this effect reached significance at a concentration of 30 μg cholesterol/mL (Figure 3(b)). Incubation of monocytes with pCRLP at similar concentrations, on the other hand, had no significant effect on MCP-1 secretion (Figure 3).

3.2. Discussion. There is increasing evidence from recent *in vitro* and *in vivo* human studies in our laboratory and others to indicate that interaction of CMRs with monocytes may contribute to atherosclerosis progression. Postprandial analysis of human leukocytes isolated after ingestion of a fat meal revealed that they take up TG-rich lipoproteins,

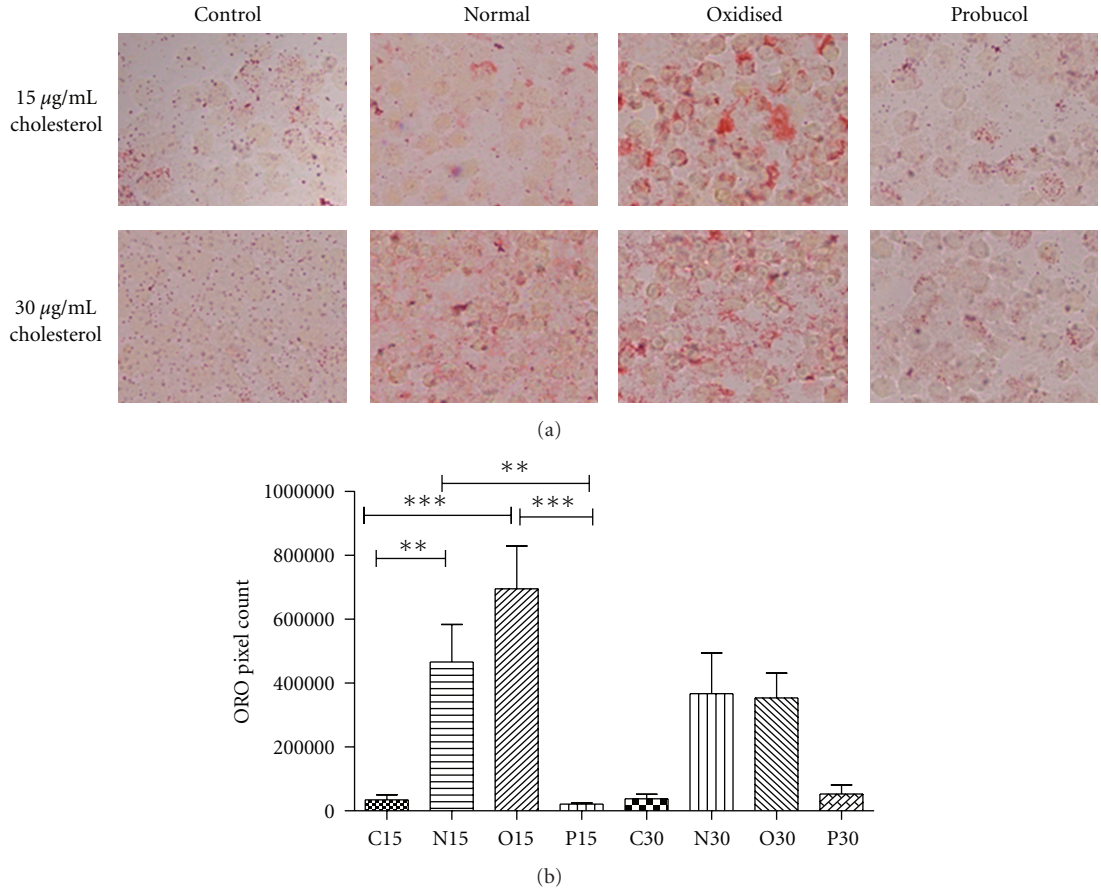

Figure 1: Uptake of native, oxidised, and probucol treated CRLP by primary human blood monocytes. Monocytes were incubated with CRLP at 37°C for 24 h before cytospin fixation and oil Red O staining. (a) Representative images (captured at magnification ×40 and enlarged). (b) Volocity quantitation (pixel count analysis) from $n = 6$ monocyte isolations. Approximately, 700 cells were examined for each condition. C15/C30 control equivalent volume to 15/30 μg/mL cholesterol, N15/N30, CRLP at 15/30 μg/mL cholesterol, O15/O30 oxCRLP at 15/30 μg/mL cholesterol, P15/P30 pCRLP at 15/30 μg/mL cholesterol. $^{**}P < 0.01$, $^{***}P < 0.001$ analysis by one-way ANOVA and Bonferroni's posttest.

resulting in increased expression of activation markers including CD11b [28]. In addition, our previous work has demonstrated that chylomicron remnants influence proinflammatory, pro-atherogenic signalling in human primary monocytes including ROS production, cytokine and chemokine expression, as well as modulating their migration towards a chemotactic gradient [30]. This is likely to be significant for development of atherosclerosis, taken together with the finding that removal of CMR from the blood is delayed in several common conditions such as obesity and type 2 diabetes [34].

The oxidative state of CMR may be influenced by a number of factors. They may be protected from oxidation by the presence of lipophilic antioxidants from the diet, they are known to carry oxidized lipids from the diet and it seems likely that they are also oxidized within the artery wall by the processes which are known to cause LDL oxidation [31, 32, 35], and the relatively large and potentially polyunsaturated fatty acid-rich CMRs particles are thought to deliver a greater oxidant load to the artery wall than LDL [36]. Furthermore, our earlier work has established that the oxidative state of CMR has profound effects on their interactions with macrophages [18, 22, 24] and thus is important for their potential atherogenicity. In the present study, we have investigated the importance of the oxidative state of CRLP on monocyte lipid accumulation and activation. The model CRLPs used have been shown to resemble physiological CMR in their size, density, and lipid composition and to be metabolised in a similar way both *in vivo* and *in vitro* in cell cultures [16, 19, 37–40]. Moreover, the maximum concentration of CRLP used (30 μg cholesterol/mL (78 μM)) is well within the range found in triglyceride-rich lipoproteins (TRL) in human plasma (postprandially; TRL cholesterol values have been reported to reach 180–250 μM [41, 42]).

Our previous findings with macrophages using both HMDM and THP-1 macrophages have demonstrated that the effects of CRLPs on lipid accumulation are inversely related to their oxidative state of the particles [17, 18, 22]. In contrast, in primary human monocytes, CRLPs protected from oxidation by the incorporation of probucol were found to inhibit intracellular lipid accumulation (Figure 1), whilst

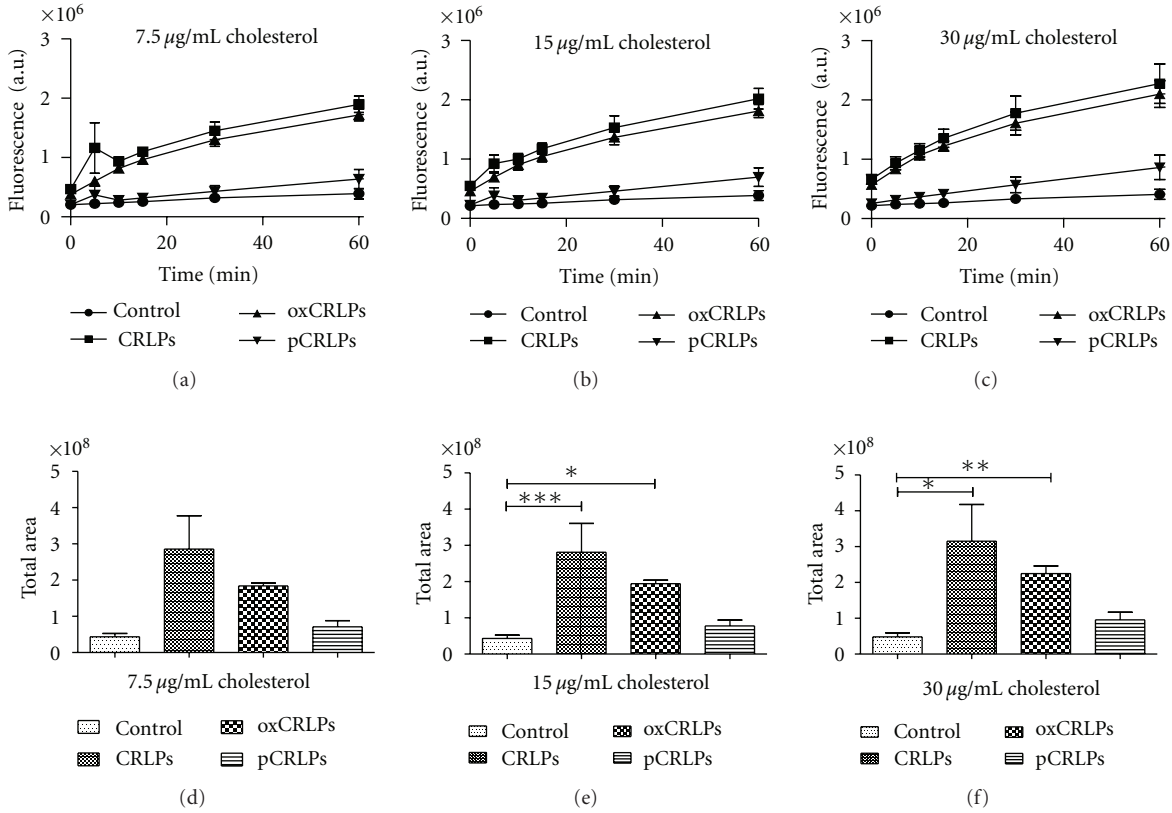

Figure 2: Generation of reactive oxygen species after coculture of CRLP with primary human blood monocytes. Monocytes were preloaded with dihydrorhodamine 1, 2, 3 before incubation with CRLPs, oxCRLPs, or pCRLPs and measurement of fluorescence (ROS production) at increasing timepoints. (a, b, c) Time course of ROS production (fluorescence) 7.5 μg/mL, 15 μg/mL and 30 μg/mL cholesterol, respectively. (d, e, f) area under curve analysis (Prism) 7.5 μg/mL, 15 μg/mL and 30 μg/mL cholesterol respectively. $n = 5$ monocyte isolations. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ paired t-test.

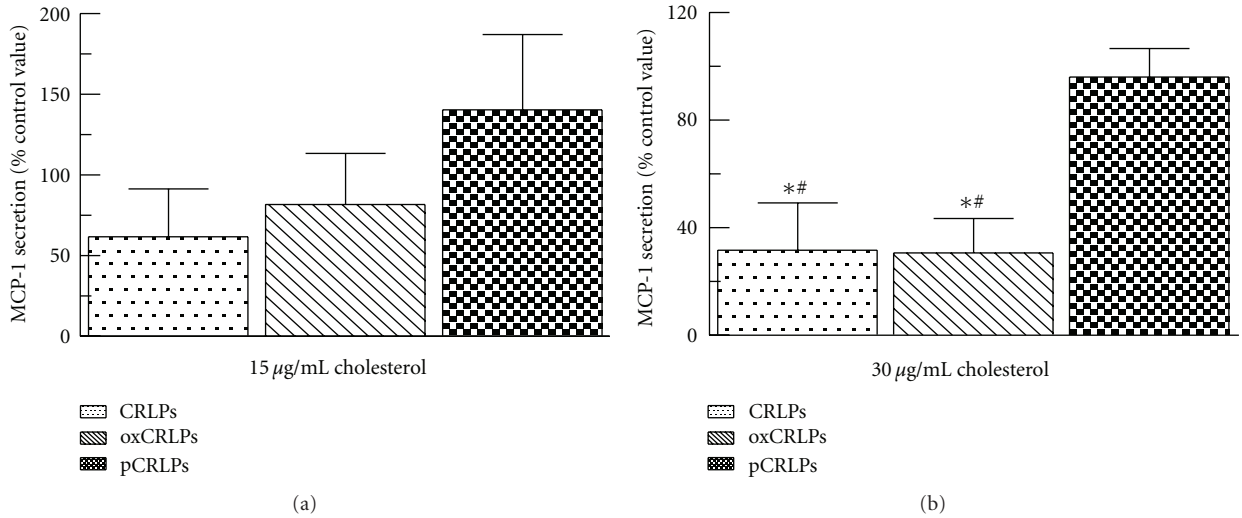

Figure 3: MCP-1 production by human primary blood monocytes after incubation with native, oxidised, or probucol-treated CRLPs. Monocytes were incubated with CRLPs, oxCRLPs or pCRLPs (a) 15 μg/mL cholesterol; (b) 30 μg/mL cholesterol) for 24 h at 37°C. After this time cells were pelleted and the supernatants were collected for analysis by ELISA. $n = 3$ monocyte isolations. $^{*}P < 0.05$ compared to control, $^{\#}P < 0.05$ compared to pCRLPs-treated monocytes. One-way ANOVA with Bonferroni's posttest.

oxCRLPs behaved in a similar manner to CRLP. Thus, prior to differentiation of monocytes into macrophages, changes in the oxidative state of CRLP have effects on intracellular lipid accumulation which more closely resemble those seen with LDL. This difference may be due to the receptor-mediated mechanisms used by the cells to take up the particles. Our previous work has suggested that the enhanced uptake of probucol-containing CRLPs by THP-1 macrophages is due to increased uptake via the LDL-receptor-related protein-1 [22]; however, the expression of this protein has been found to be low in monocytes and induced only on differentiation into macrophages [43]. Thus, reduced expression of LRP-1 in monocytes may account for both the lower uptake of pCRLP as compared to CRLP in these cells and for the differing effects of the presence of probucol on uptake of the particles in monocytes as compared to macrophages.

ROS production is stimulated in monocytes as a defence against infection and during other inflammatory reactions [3–5]. Human monocytes have been reported to generate ROS when exposed to oxidised LDL [44], and CMRs have been found to increase ROS production in THP-1 cells [30]. In agreement with our earlier study [30], CRLP treatment of monocytes caused rapid and prolonged generation of ROS (Figure 2). Although this effect was not enhanced when the particles were oxidized, it was abolished completely when pCRLPs were used. These results suggest that the presence of antioxidant in CMRs inhibits their induction of monocyte ROS production.

Human peripheral blood monocytes are known to increase the secretion of MCP-1 in response to inflammation [4]. We have found previously, however, that CRLP cause a marked decrease in MCP-1 secretion by human monocytes [30], and we have also demonstrated similar effects in macrophages using HMDMs and THP-1 cells [21, 22]. Evaluation of the effects of the oxidative state of the CRLPs in macrophages showed that oxCRLPs caused a similar reduction to that observed with CRLPs, but, when the particles were protected from oxidation with probucol, the effect was significantly reduced [22]. The results of the present study indicate that monocytes behave like macrophages in the response of MCP-1 secretion to changes in the oxidative state of CRLP, with oxCRLP and CRLP decreasing production to a similar extent, while the incorporation of antioxidant into the particles abolishes the effect (Figure 3). However, in our earlier monocyte study [30], we demonstrated that a decrease in medium concentrations of MCP-1 after exposure of monocytes to CRLP increased cell migration towards a higher concentration of the chemokine. This finding suggests that CMR may cause an increase in the chemotactic gradient of MCP-1 across the endothelium because of reduced MCP-1 secretion by monocytes in the blood vessel lumen microenvironment thus having a promigratory effect on circulating monocytes. Our present results, therefore, suggest that the presence of antioxidants in CMR may decrease their propensity to enhance monocyte chemotaxis.

Previous studies with oxLDL have demonstrated that its oxidative state is important for the reported proinflammatory, proatherogenic effects in macrophages [5, 26, 45]. Interestingly, although clinical trials with dietary supplementation of antioxidants have proved disappointing, [46] *in vitro* antioxidants have shown efficacy. Treatment of endothelial cells with the antioxidant flavonoid, luteolin, protects them from the effects of oxLDL effects by downregulation of the lectin-like oxidised LDL receptor [47]. Similarly, the citrus-derived flavonoid nobiletin has recently been reported to inhibit monocyte to macrophage differentiation and scavenger receptor activity in THP-1 cells via inhibition of PKC [48]. Although we cannot completely rule out the possibility that the effects observed in the present work are specifically due to the presence of probucol, rather than protection of the particles from oxidation, our previous work with macrophages showing that probucol and lycopene, a chemically unrelated antioxidant, have remarkably similar effects on lipid accumulation suggest that this is not the case. Thus, the present work provides evidence to suggest that antioxidants from the diet carried in CMR may help to protect against the effects of these lipoproteins in promoting the activation of human primary monocytes, but further studies are required to substantiate this conclusion.

Previous work in our laboratory has shown that downregulation of NF-κB activation is involved in the inhibition of proinflammatory cytokines secretion by CRLPs [21]. It seems likely, therefore, that NF-κB is involved in the modulation of monocyte activation by CMRs, and this is supported by the finding from our previous study that CRLP-stimulated monocyte ROS production is mediated via NF-κB [30]. Thus, there may be early activation of this pathway followed by later inhibition of de novo NF-κB synthesis, leading to downregulation of constitutive MCP-1 expression.

4. Conclusions

This study demonstrates that the changes in the oxidative state of CRLP influence their effects on peripheral blood monocytes. Thus, protection of the particles by the incorporation of the antioxidant probucol decreases the induction of lipid accumulation and ROS production in the cells observed with CRLP and oxCRLP but abolishes the suppressive effect on MCP-1 secretion. The results support the hypothesis that CMR play a role in the increase in monocyte activation which contributes to early atherosclerotic lesion formation and suggest that dietary antioxidants carried in CMR may reduce or prevent some of the potentially inflammatory effects of the lipoproteins on these cells.

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Review Article

Understanding Postprandial Inflammation and Its Relationship to Lifestyle Behaviour and Metabolic Diseases

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Postprandial hyperlipidemia with accumulation of remnant lipoproteins is a common metabolic disturbance associated with atherosclerosis and vascular dysfunction, particularly during chronic disease states such as obesity, the metabolic syndrome and, diabetes. Remnant lipoproteins become attached to the vascular wall, where they can penetrate intact endothelium causing foam cell formation. Postprandial remnant lipoproteins can activate circulating leukocytes, upregulate the expression of endothelial adhesion molecules, facilitate adhesion and migration of inflammatory cells into the subendothelial space, and activate the complement system. Since humans are postprandial most of the day, the continuous generation of remnants after each meal may be one of the triggers for the development of atherosclerosis. Modulation of postprandial lipemia by lifestyle changes and pharmacological interventions could result in a further decrease of cardiovascular mortality and morbidity. This paper will provide an update on current concepts concerning the relationship between postprandial lipemia, inflammation, vascular function, and therapeutic options.

1. Introduction

Atherosclerosis is the primary cause of death in the world [1]. Classical risk factors such as smoking, hypertension, fasting hyperlipidemia, insulin resistance, increased body fat mass, and unfavourable body fat distribution are strongly interrelated and can often be found in one and the same subject. Subjects with fasting hypertriglyceridemia usually have elevated postprandial lipids due to the close correlation of fasting and postprandial triglycerides (TG) [2]. Postprandial lipemia has gained interest because of recent reports showing that nonfasting TG independently predict the risk for atherosclerosis [3, 4] and are possibly even stronger predictors of cardiovascular disease (CVD) than fasting TG [3, 5].

Atherosclerosis is considered a low-grade chronic inflammatory disease [6], and both the postprandial phase and chronic disease states such as the metabolic syndrome are associated with increased inflammation. This paper outlines recent developments in the understanding of postprandial inflammation and its relationship with vascular function, metabolic diseases, and lifestyle behaviour.

2. Metabolism of Postprandial Lipemia

Dietary fat is absorbed in the intestine and secreted into lymph by enterocytes in TG-rich chylomicrons. Once in the circulation, chylomicrons rapidly undergo hydrolysis to produce cholesterol-dense lipoprotein remnants which are taken up by the liver [7, 8]. After a fatty meal, exogenous fatty acids are delivered to the liver by chylomicron remnants and may then be reassembled and returned to the blood in very low-density lipoproteins (VLDL) [9].

The hypertriglyceridemia observed postprandially is due to raised concentrations of chylomicrons, VLDL, and their respective remnants, collectively known as triglyceride-rich lipoproteins (TRLs).

People in the Western world are nonfasting for most of the day, consequently leading to a continuous challenge of the endothelium by atherogenic lipoprotein remnants [10, 11]. The exogenous chylomicrons and endogenously produced VLDL share the same metabolic pathway, for example, endothelium-bound lipoprotein lipase (LPL), which hydrolyzes TG into glycerol and fatty acids. In the postprandial phase, due to limited LPL availability, competition at the level of this enzyme will occur resulting in accumulation of TRLs. This competition is most likely when fasting hypertriglyceridemia is present. The increased levels of free fatty acids (FFAs) as a result of a hypercaloric diet are regarded as one of the key etiologic components of the metabolic syndrome, type 2 diabetes (T2DM), and obesity [12, 13].

3. Residual Risk of Cardiovascular Disease after LDL Cholesterol Lowering

Based on results from large clinical trials, lipid management for reducing the risk for CVD has been typically focused on reducing LDL-C by statin therapy [14–18]. Despite aggressive LDL-C lowering by statin therapy, approximately two-thirds of all CVD events remain. These "residual" events appear to be independent of the LDL-C and in recent years have gained momentum as a concept of "residual risk" of CVD. Interestingly, statins do reduce postprandial lipemia and also have an effect on complement, but they do not affect TG sufficiently to be of clinical relevance in hypertriglyceridemic conditions [2, 19–22]. Interestingly, this "residual risk" has been found to be greater for treated patients with diabetes or the metabolic syndrome than in untreated patients without these conditions [23, 24]. One could interpret these observations to infer that statin therapy, resulting in LDL-C lowering, does not necessarily bring the relative CVD risk in patients suffering from diabetes and metabolic syndrome to the level of nondiabetics and patients without metabolic syndrome. Consequently, the current model we propose is that the "residual risk" hypothesis of atherosclerosis is not just dependent on circulating concentrations of LDL-C but is equally dependent on remnant lipoprotein concentrations and perturbations in the arterial vessel wall that influence the rate of arterial lipoprotein retention. The potential impact of the "residual risk" hypothesis is perhaps most appreciated during conditions of increased atherosclerotic risk. For example, subjects with insulin resistance, T1DM, or T2DM showed raised plasma concentrations of fasting remnant lipoproteins and an ensuing impairment in postprandial lipoprotein metabolism [25, 26]. The remnant lipoproteins are able to penetrate arterial tissue and become entrapped within the subendothelial space [27]. It has also been demonstrated that remnant lipoproteins can induce macrophage lipid loading, which is a hallmark feature of early atherogenesis [28–30]. Moreover, raised fasting concentrations of apoB48, the specific protein of chylomicrons and their remnants [31], have been shown to be elevated in patients with obesity, insulin-resistance, and T2DM [32–35]. Numerous studies have shown that fasting levels of remnant lipoproteins can predict impaired metabolism of chylomicrons, particularly in those at risk of CVD [36–38]. Under experimental conditions, some studies have suggested that the small LDL particles may have a higher rate of delivery but in turn efflux more readily from arterial tissue compared to remnant lipoproteins [39, 40]. However, despite many studies showing atherogenic effects of impaired postprandial lipemia, it remains uncertain whether lowering postprandial lipemia would reduce CVD risk or if TG are merely a marker of other metabolic abnormalities [41]. But it is certain that postprandial lipemia, obesity, insulin resistance, inflammation, and vascular function and atherogenesis are closely related with each other.

4. Postprandial Lipemia and Vascular Integrity

Coronary arteries are characterized by tightly apposed endothelial cells with significant expression of tight junction proteins [42]. In healthy vessels, the coronary endothelium prevents the diffusion of large or hydrophilic molecules, thereby minimising extravasation of systemically derived potentially inflammatory agents and macromolecules. However, some lipoprotein transport including remnants of TRLs occurs across intact and functional endothelium via nonspecific transcytotic processes [40]. This phenomenon is predominantly non-pathogenic, because the lipoproteins are internalized via receptor processes or passage through the basal laminae and exit via the vasa vasorum. However, experimental evidence suggests that prolonged retention of TRLs as a consequence of binding to extracellular matrices and proteoglycans can stimulate chemotaxis and activation of circulating inflammatory cells (Figure 1) [43].

Dietary-lipotoxicity is a term commonly used to broadly describe processes leading to end-organ damage following excess exposure to particular lipids [44]. First identified in the context of fat-induced insulin resistance, the process has since been implicated in a range of chronic diseases and inflammatory disorders. Endothelial cells may be particularly susceptible to the effects of dietary lipids associated with TRLs because of the significant level of lipoprotein processing that occurs via interaction with hydrolytic lipases and, thereafter, the constant exposure to plasma fatty acids and cholesterol. Animal feeding studies have shown that saturated fatty acids and cholesterol-enriched diets increase protein oxidation and lipid peroxidation and significantly alter cell membrane phospholipids and lipid raft composition, key regulators of inflammation [45, 46]. Excess cholesterol can cause mitochondrial dysfunction and trigger apoptotic pathways [47]. Other mechanisms for dietary fat-induced alterations in cell function include stimulation of NADPH-oxidase-derived reactive oxygen species or modulation of mTOR (mammalian target of rapamycin),

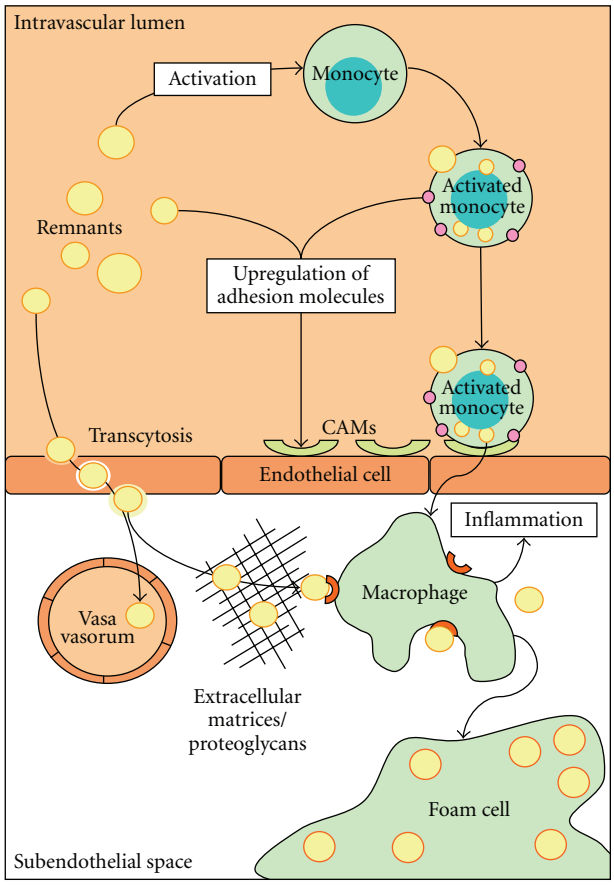

FIGURE 1: Concept of the initiation of atherosclerosis by remnant lipoproteins: remnants enter the subendothelial space via nonspecific transcytotic processes. This is often a nonpathologic process, because the remnants leave the subendothelial space again via the vasa vasorum. However, retention of remnants may occur in the presence of proteoglycans and excess extracellular matrices. Remnants can be easily taken up by macrophages, in contrast to LDL, which need to become modified first. Circulating remnants themselves also contribute to the presence of subendothelial macrophages. Monocytes can bind and take up remnants, which stimulates the monocytes to become activated. Subsequently, activated monocytes express adhesion molecules on the outer membrane and stimulate the expression of endothelial cellular adhesion molecules (CAMs), which allows monocytes to home on the endothelium and migrate into the subendothelial space. Finally, the macrophages change into highly atherogenic foam cells when lipid uptake exceeds lipid efflux.

a key signal transduction protein that regulates vascular endothelial fenestration [48]. Many other factors have been suggested to participate in the generation of oxidative stress. Paraoxonase 1 (PON-1), a potent antioxidant closely associated with HDL-C, seems to be a key player [49]. During postprandial lipemia, HDL-C tends to decrease, impairing the reverse cholesterol transport and reducing the anti-inflammatory properties of HDL-C [50], again providing an extra atherogenic mechanism for postprandial lipemia.

Commensurate with dietary fat modulation, the particle phenotype of lipoproteins determines the susceptibility to subendothelial retention. The heparin sulphate proteoglycans that bind apo B lipoproteins may have greater affinity for TRL remnants because of cooperative apolipoprotein binding domains principally between apo B and apo E and exacerbates as a consequence of diabetes [51]. Moreover, apo E facilitates unabated uptake of remnant lipoproteins by macrophages via alternate pathways without the requisite of lipoprotein modification such as oxidation [52, 53].

Nitric oxide (NO) is also one of the key players of endothelium-derived factors, which influences vasomotion, permeability, proliferation, and vascular smooth cell migration [54]. NO-mediated endothelial-dependent vascular relaxation has been shown to be impaired by remnant lipoproteins in studies with isolated vessel segments from rats and pigs *in vitro* [55]. In human subjects with the metabolic syndrome, but also in healthy subjects, elevated fasting and postprandial TG have been related to increased carotid intima-media thickness (IMT) [56] and reductions in NO-dependent postischemic flow-mediated dilation (FMD) of the brachial artery [57, 58]. This reduction of FMD correlated with TG and FFA concentrations and was reversible when TG concentrations decreased at the end of the oral fat loading test [57]. Furthermore, postprandial TRLs have been shown to induce the expression of leukocyte adhesion molecules on the endothelium, facilitating recruitment of inflammatory cells [59] and remnant lipoproteins have been found to activate endothelial cells by upregulating COX-2 expression and activating intracellular signaling pathways controlled by nuclear factor-kappaB and mitogen-activated protein kinases [60].

5. Triglyceride-Rich Lipoproteins and Inflammation

Many inflammatory markers, such as C-reactive protein (CRP), leukocyte count, and complement component 3 (C3), have been associated with CVD [61–66]. Furthermore, several studies with animal models showed reduced plaque formation [67, 68] and prevention of endothelial dysfunction [69], when adherence of leukocytes to the endothelium was prevented. These findings support the theory that atherogenesis, in part, starts with leukocyte-endothelium interaction and adherence. Obligatory for this adherence is a cytokine-controlled sequential upregulation of selectins and adhesion molecules on activated leukocytes and endothelial cells [70].

Van Oostrom et al. have shown that postprandially, when TG and glucose rise, neutrophil counts increase with concomitant production of pro-inflammatory cytokines and oxidative stress; and that these changes may contribute to endothelial dysfunction [71, 72]. Furthermore, TG and glucose are able to induce leukocyte activation, as has been shown *in vitro* [73, 74] and *ex vivo* in hypertriglyceridemic patients [75]. In healthy volunteers and in patients with premature CVD, postprandial lipemia has been associated with the upregulation of leukocyte activation markers [22, 76]. Fasting leukocytes of patients with CVD have an increased lipid content when compared to controls, and it has been

suggested that this is due to uptake of chylomicrons [77]. Furthermore, uptake of remnant lipoproteins by primary human monocytes has been demonstrated in experiments *in vitro* [74]. Leukocytes are also able to take up retinyl esters, as markers of intestinally derived TRLs [78]. Recently, we have shown that apo B binds to neutrophils and monocytes and that postprandial leukocytes transport dietary fatty acids [79]. This opens the possibility that direct activation of leukocytes may occur in the blood by interaction with chylomicrons and their remnants (Figure 1).

Another inflammatory pathway related to CVD and lipid metabolism is the complement system. The C3/acylation stimulating protein- (C3/ASP-) system has been recognized as a regulator of adipose tissue fatty acid metabolism [80]. ASP is identical to the desarginated form of the C3 split-product C3a (C3a-desArg), which is immunologically inactive. The C3/ASP pathway stimulates re-esterification of FFA into TG in adipocytes, reduces adipocyte FFA production by inhibiting hormone sensitive lipase and stimulates glucose uptake by adipocytes, fibroblasts, and muscle cells [80]. C3 is a strong predictor of myocardial infarction [64], and it has been positively associated to obesity, CVD, insulin resistance, the metabolic syndrome [81], fasting and postprandial TG, and hypertension [2, 64]. Complement components have been shown to colocalize with CRP in atherosclerotic plaques [82] and complement activation also plays a role in the induction of tissue damage after myocardial infarction [83]. Moreover, chylomicrons are the strongest *in vitro* and *in vivo* stimulators of adipocyte C3 production via activation of the alternative complement cascade [66, 84]. A postprandial C3 increment after a fat meal has been shown in healthy subjects, patients with CVD, and patients with familial combined hyperlipidemia [2, 63, 66]. Moreover, this postprandial increment has been related to TG and FFA metabolism [85].

6. Metabolic Syndrome and Insulin Resistance in Relation to Atherosclerosis and Postprandial Lipemia

Insulin resistance has also been shown to be associated with impaired vasodilatation, increased oxidative stress and increased concentrations of FFAs, vasoconstrictors, cell adhesion molecules, cytokines, and several other mediators of low-grade inflammation and thrombogenesis [86]. Insulin resistance increases the risk for CVD severalfold compared to the normal population; however, the underlying mechanisms are not completely defined [87]. Insulin resistance often clusters with elevated blood pressure, obesity, central obesity, elevated TG, and low HDL-C. However, whether hyperinsulinemia itself is indeed an independent predictor of CVD has often been debated [87]. A recent meta-analysis by Ruige et al. showed a weak positive association between high insulin levels and CVD events [88]. Another meta-analysis involving 87 studies, which included 951,083 patients based on the definitions of metabolic syndrome by the 2001 National Cholesterol Education Program (NCEP) and 2004 revised National Cholesterol Education Program (rNCEP) demonstrated a 2-fold increase in cardiovascular outcomes and a 1.5-fold increase in all-cause mortality in subjects with the metabolic syndrome [89]. According to the NHANES III data, subjects with metabolic syndrome but without diabetes had a significantly increased prevalence of CVD [90]. However, recently, it was shown that the metabolic syndrome could not improve prediction of intima media thickness progression compared to the sum of its risk components [91]. It is evident that postprandial lipemia is prevalent during conditions of obesity and insulin resistance and may contribute to increased progression of CVD. However, a significant clinical dilemma still exists in diagnosing the early phases of the metabolic syndrome (i.e., prediabetes) and how this impacts on relative risk of CVD. In part, this has been impaired by the continued emphasis on LDL-C, which is often normal during early T2DM, leading to undetected yet insidious progression of CVD [92, 93]. Indeed, it is interesting to note that the recent revision by the IDF (International Diabetes Federation) has defined the metabolic syndrome independent of LDL-C concentrations [94]. In general, in clinical practice, the positive effects of LDL-lowering therapy on atherosclerosis and CVD are nowadays undisputable. While these efforts are well documented, much less is known about the clinical benefits of treating postprandial lipemia, despite increasing evidence supporting a causal role between remnant lipoproteins and the development of CVD [95]. Clinical studies have so far failed to provide a definitive association between impaired postprandial lipoprotein metabolism and the very early phases of insulin resistance and corresponding risk indices. Thus, animal models have to offer further characterization of the early stages of metabolic syndrome in order to understand the metabolic and postprandial profile of this condition. Despite a greater emphasis on the study of CVD risk in the metabolic syndrome, there remains a lack of well-characterized prediabetic models in order to investigate the role of postprandial lipoprotein metabolism in the development of atherosclerosis.

7. Diet, Lifestyle, Pharmacotherapy, and Postprandial Lipemia

Postprandial hyperlipidemia has many negative effects on vascular integrity, inflammation, and fatty acid metabolism but can be positively influenced by diet and lifestyle behaviour. Since postprandial lipemia is a physiological response to a fatty meal, it could be predicted that it would be influenced by the amount and type of fat in the diet, and there is strong evidence to support this [96, 97]. However, in recent years, it has become clear that other lifestyle factors, including dietary protein, fibers and micronutrients, alcohol consumption, exercise, and smoking also play a significant role in the regulation of postprandial lipemia [96]. Postprandial hyperlipidemia may be a link between lifestyle choices and the current alarming rise in the incidence of obesity, insulin resistance, T2DM, and CVD [98]. A summary of the positive and negative effects of lifestyle factors and metabolic diseases on postprandial lipemia is shown in Figure 2.

Postprandial lipemia is evident after a fat meal containing >30 g fat and the rise in plasma TG is dose dependent up to about 80 g [96, 99]. Since the average content of Western style meals is 20–40 g fat and 3-4 meals/day are typically consumed, it can be concluded that postprandial lipemia is likely to be present for 18 h/day in the Western population [100]. A single fatty meal causes changes in TRL particle characteristics, such as their size, number, and apolipoprotein composition, which depend on the fatty acid composition of the food. The most pronounced lipemia judged by these criteria is caused by a meal containing saturated fatty acids (SFAs), which are found in high amounts in animal fat, followed by monounsaturated fatty acids (MUFA), the main fatty acids in olive oil, with polyunsaturated fatty acids (PUFA), which are found in vegetable (n-6 PUFA) and fish (n-3 PUFA) oils, causing the least pronounced effect [96]. n-3 PUFA have also been shown to cause a lower rise in postprandial lipemia compared to the other types of fat [96, 101, 102]. While acute studies provide useful information, changes in dietary habits need to be sustained in the long term for beneficial effects on health. Both chronic intake and dietary supplementation of n-3 PUFA for periods varying from 4 weeks to 6 months have been shown to decrease postprandial hyperlipidemia due to decreased production of TRLs [103–106]. The effects of fatty acids other than n-3 PUFA on postprandial lipemia are less well defined, but generally, MUFA or n-6 PUFA as compared to SFA have been found to be more beneficial [96, 107, 108].

In addition to fat, the type of dietary proteins and carbohydrates may also influence postprandial lipemia. Lean red meat, soy protein, casein, and whey protein have all been associated with a reduced postprandial lipemic response [96, 107, 109], as have indigestible carbohydrates (i.e., dietary fiber) in the form of oat bran, wheat fiber, wheat germ, or psyllium husk [96, 101, 110]. Digestible carbohydrates, on the other hand, appear to have little effect [111] except for fructose which may enhance the postprandial lipemic response if more than 50 g per day are consumed [112].

Besides macronutrients like fats, carbohydrate, and protein, the diet contains micronutrients including vitamins, carotenoids, plant sterols, and polyphenols found in fruit and vegetables and in beverages such as green tea and red wine, and these are believed to contribute to the protective effect against CVD [113]. Polyphenols in green tea and strawberries have been reported to reduce postprandial lipemia in hyperlipidemic individuals [114, 115]. In addition, micronutrients in olive oil have been found to reduce postprandial lipemia [116]; however, no evidence for an effect of plant sterols on postprandial lipemia was found in a study with patients on lipid lowering therapy [117].

In addition to changing to a healthier diet, weight loss and increased physical activity are effective lifestyle interventions which reduce postprandial lipemia [118–120]. Exercise before a fat meal, even if only low-to-moderate volume, has been found to decrease postprandial lipemia in many studies [120–125]. Moreover, moderate and high exercise bouts appear to be equally beneficial [126], and a recent meta-analysis of 16 studies concluded that exercise in short bouts is as effective as continuous exercise in lowering postprandial blood TG concentrations [127]. Low-volume exercise, however, has been reported to be ineffective in smokers [128], and Bloomer et al. have also reported recently that long-term high-volume exercise had no effect on postprandial lipemia in young, healthy individuals [129]. Combining increased physical activity with dietary changes such as increased n-3 PUFA intake has been found to have a synergistic effect in reducing postprandial lipemia in active individuals [96]. The reduction in postprandial lipemia caused by exercise is believed to be due to increased clearance of TRLs, which is at least partially mediated by an increase in LPL activity [96]. However, current evidence suggests that it is not accompanied by a decrease in the associated postprandial inflammation, as assessed by markers such as C reactive protein, IL-6, or adhesion molecules [121, 123, 124].

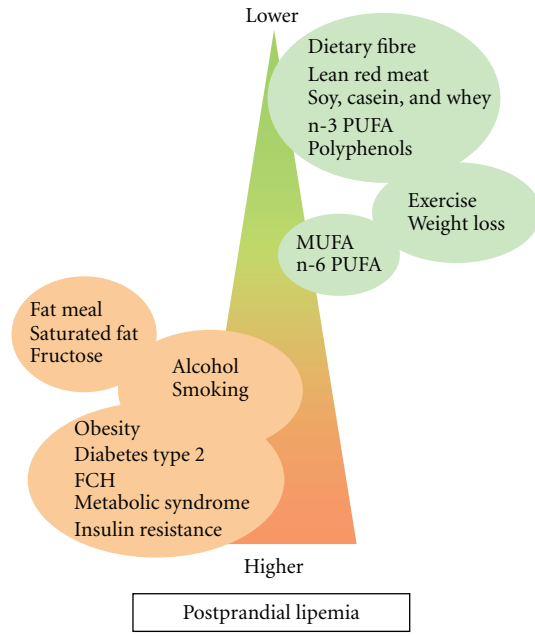

Figure 2: Influence of metabolic diseases and lifestyle factors on postprandial lipemia: factors listed in green circles reduce postprandial lipemia, whereas the factors in red have detrimental effects on postprandial lipemia.

In addition to daily meals and exercise, alcohol and smoking also influence postprandial lipemia. A study by Sharrett et al., which included >600 subjects with or without CVD, demonstrated that smoking and alcohol consumption as well as the diet are good predictors of postprandial lipid levels [100]. Despite considerable evidence that low-to-moderate alcohol intake protects against CVD [130], both ethanol and red wine have been shown to cause a marked increase in postprandial lipemia when added to a test meal [96, 131]. Habitual smokers also have increased postprandial lipemia, and this is thought to be due to impaired clearance of chylomicrons and their remnants [132].

Besides lifestyle interventions, no large improvements have been made in treating postprandial lipemia with pharmacotherapy. Statins are highly effective in reducing LDL cholesterol, but they do not affect TG sufficiently to

be of clinical relevance in hypertriglyceridemic conditions. However, rosuvastatin is able to reduce the postprandial proinflammatory and procoagulant changes in subjects with CVD [22]. In addition, a decrease in hepatic FFA flux has also been reported [133]. These independent effects from rosuvastatin may protect against CVD when hyperlipidemia is present. In contrast to statins, pharmacotherapy with fibrates is effective in lowering TG concentrations. Despite hypertriglyceridemia being common in the Western population, fibrates are used in only 3.6% of hypertriglyceridemic subjects [134]. Controversy remains in the effectiveness of fibrates on cardiovascular morbidity and mortality. Recently, a large meta-analysis of fibrates with 45,058 participants was performed [135]. This study showed a modest but significant relative risk reduction of 10% for major cardiovascular events and 13% reduction in coronary events, but mortality remained unaltered. While certain drugs are beneficial for improving insulin resistance and potentially postprandial lipemia, in specific groups of patients, the effects on chylomicron and remnant metabolism may be detrimental. For example, rosiglitazone increases postprandial accumulation of atherogenic remnants in HIV-infected patients with lipodystrophy [136]. Therefore, when evaluating the effects of specific pharmacotherapeutic interventions on postprandial lipemia, detailed information on all aspects of the potentially harmful situations will be needed. At this stage, there is no data available on comparative studies regarding lifestyle modification versus lipid lowering therapy on the modulation of postprandial lipemia.

8. Conclusions

A residual risk for CVD remains despite aggressive LDL-C lowering by statins, which can partly be explained by postprandial hyperlipidemia, which leads to several metabolic dysfunctions and dietary lipotoxicity via different several mechanisms. First of all, TRLs are able to penetrate the arterial wall leading to endothelial lipid deposits, attraction of monocytes within the subendothelial space, production of inflammatory markers, and oxidative stress. Secondly, obesity worsens insulin resistance which further increases postprandial lipemia, consequently resulting in a vicious circle. Lifestyle interventions like the type of diet, cessation of smoking, and weight loss are effective methods to reduce postprandial lipemia and its related dietary lipotoxicity. Statins also have a beneficial effect on postprandial lipemia. However, at this stage, there are no data available comparing the magnitude of lifestyle interventions with pharmacotherapy on chylomicron metabolism and reduction of CVD.

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Review Article

The Effect of Alcohol on Postprandial and Fasting Triglycerides

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Alcohol has a significant additive effect on the postprandial triglyceride peak when it accompanies a meal containing fat, especially saturated fat. This results from a decrease in the breakdown of chylomicrons and VLDL remnants due to an acute inhibitory effect of alcohol on lipoprotein lipase activity. Furthermore, alcohol increases the synthesis of large VLDL particles in the liver, which is the main source of triglycerides in the hypertriglyceridemia associated with chronic excessive alcohol intake. In case of chronic consumption, lipoprotein lipase activity seems to adapt itself. The effect of alcohol on adipose tissues is less clear. Sometimes, a severe hypertriglyceridemia induced by alcohol (SHIBA) can be observed, especially in patients with type 2 diabetes mellitus and/or obesity increasing the risk of pancreatitis.

1. Introduction

Hypertriglyceridemia (HT) may result from genetically determined disturbances in lipid metabolism but may also be secondary to conditions such as obesity, diabetes mellitus, hypothyroidism, the nephrotic syndrome, and the use of medication. Quite a number of drugs can be associated with HT including β-blockers, steroids, diuretics, oestrogens, immunosuppressants, cytostatics, and antiviral drugs [1]. Although its health effects are continuously a subject of debate, alcohol (ethanol) can be added to this list [2, 3].

Since both food and alcohol are known to affect lipid metabolism, a number of studies have been directed to their combined effect. This paper will focus on triglycerides after the consumption of such mixed meals as well as on the observation that alcohol may induce a very severe form of HT, which may be of clinical relevance with an increased risk of pancreatitis [4].

2. Alcohol and Triglycerides in Animal Studies

The metabolic effects of alcohol on the liver and lipid metabolism are known for many years and have been extensively studied by Lieber and many of his coworkers [5]. Although the administration of one dose of a diet containing alcohol (3 g/kg) given to rats produces a significant increase in mesenterial lymph flow, lipid output, and incorporation of dietary fat into lymph lipids, this does not result in HT [6]. By contrast, previous feeding of alcohol for several weeks results in postprandial hyperlipemia after a single dose of the mixed meal and even after such a dose without the alcohol component. Baboons fed with a liquid diet containing 50% of energy as ethanol for 5–16 months develop HT and alcoholic liver injury (fatty liver) [7]. This HT results from an increased production of large VLDL particles by the liver, while the splanchnic extraction of TG from chylomicron- and VLDL-remnants is secondarily enhanced. In another rat experiment, Daher et al. confirmed a rise in plasma triacylglycerol and chylomicron concentrations after ethanol ingestion but also found a decreased chylomicron size with a change in cholesterol and phospholipid content indicating enhanced liver bile secretion [8]. In case the animals were put on a moderate alcohol diet for a period of ten weeks, their postprandial HT and hyperchylomicronemia were less pronounced. This is probably related to an adaptive increase of lipoprotein lipase (LPL) activity in case of chronic moderate alcohol consumption [9]. In contrast, an acute ingestion of ethanol lowers LPL activity. Another mechanism which may contribute to postprandial HT is a decrease of lipogenesis and glucose oxidation in adipose tissue, as shown in rats after chronic ethanol feeding [10]. Kang et al. [11] studied triglyceride turnover in white adipose tissue and showed that

chronic alcohol consumption inhibits the antilipolytic action of insulin. It has also been shown that the lipemic response to alcohol is related to the stage of liver disease, since in cirrhosis, in contrast to steatosis, fasting lipid response is neglectable, but postmeal chylomicron response is increased [12].

Apart from serum HT, alcohol may also induce accumulation of triacylglycerols in the liver, leading to steatosis hepatis. This influence of alcohol can be partly explained by impairment of AMP-activated protein kinase (AMPK), which enzyme plays a central role in hepatic fatty acid metabolism [13]. Several dietary regimes have proven to be able to influence this process of alcohol-induced fatty liver including medium-chain triglycerides and fish oil [14, 15].

Interestingly, the consumption of alcohol stimulates the intake of fat, while dietary fats stimulate the consumption of alcohol, a vicious cycle probably mediated by hypothalamic peptides [16].

3. Triglycerides after Mixed Meals in Humans

When normal healthy volunteers consume wine, in total 30 g of alcohol, during a standard diner, postprandial TG, measured one hour after intake, increase by 15.3%, but after overnight fasting, values have returned to normal [17]. This effect is clearly related to alcohol and not to the type of drink, since in a similar experiment, no differences were found between wine, beer, and spirits [18]. In another experiment of these investigators, they showed that TG levels reach their peak three hours after dinner with the same response in men and women [19]. Even so, the response is not different in men with a low-risk profile for cardiovascular disease compared to men with a high-risk profile [20].

Since both alcohol and exercise have an effect on postprandial lipemia and TG clearance, their combined effect has been studied. El-Sayed and Al-Bayatti [21] studied plasma TG concentrations after exercise followed by a diet with and without alcohol. In the control trial, when subjects consumed a standardized lunch after their 35 minutes of exercise (VO2max 70%), TG showed no significant change. However, when alcohol was consumed with the lunch, TG concentration increased substantially 5 hours during recovery. The mechanism responsible for this TG rise was not studied, but inhibition of LPL activity by alcohol may play a role. On the other hand, Hartung et al. were able to show that alcohol-induced increases in postprandial lipemia and retardation of TG clearance occur in inactive men but not in exercise-trained subjects [22].

Even if the diet contains a lot of fat resulting in high postprandial TG levels, the addition of alcohol still has a significant additive effect. Franceschini et al. [23] performed an experiment in which normolipemic subjects either consumed 70 g of fat or this amount of fat in combination with 40 g of ethanol. Four to six hours, after the fat intake TG levels rose by 70% but after the intake of both fat and ethanol by 180%. A similar study was designed by Pownall [24] in which three different fat loads were given to normal subjects with and without alcohol. The fat loads consisted of saturated fat, polyunsaturated fat, or polyunsaturated fat with omega-3 fatty acids. Preprandial alcohol increased postprandial lipemia, an effect that was most profound with the saturated fat load. Alcohol had no effect on the plasma concentrations of free fatty acids derived from peripheral tissue but appeared to decrease the plasma concentration of free fatty acids from dietary origin. These data are highly suggestive for an impairment of chylomicron hydrolysis due to inhibition of LPL. Fielding et al. [25] also found no arguments for an effect of alcohol on the release of nonesterified fatty acid into the circulation.

Because of the increased risk of cardiovascular diseases in diabetes patients and the possible cardioprotective effect of alcohol, Dalgaard et al. [26] studied the effect of a mixed meal on postprandial lipemia and incretin levels in type 2 diabetes patients. Early in the postprandial phase, alcohol suppresses the incretin responses and increases the late postprandial TG levels. This alcohol-induced suppression of the incretin response resulting in lower insulin levels may contribute to the impaired TG clearance in type 2 diabetes patients but may also be operative in nondiabetics.

4. Fasting Triglycerides and Regular Alcohol Consumption

In contrast to moderate alcohol consumption, excessive intake may cause HT even in the fasting state [5, 17]. This effect seems to be more pronounced in African-Americans than in white Americans [27]. When regular and binge drinkers cut down their alcohol intake, a more or less similar drop (0.22 and 0.26 mmol/L, resp.) in fasting TG is observed, indicating that HT is more related to the amount of alcohol consumed than to the pattern of drinking [28]. Pownall et al. [29] studied the effect of the consumption of two alcoholic drinks in the fasting state in patients with mild hypertriglyceridemia (2.3–8.5 mmol/L) in comparison with normal lipemic individuals. At six hours (peak), TG concentration increased only 3% in the HT group and 53% in the nonhypertriglyceridemic group. They conclude that alcohol intake alone in not an important determinant of plasma TG concentration in individuals with HT.

On the other hand in a recent study analyzing the underlying disorders in patients with severe HT, alcohol proved to be of dominant importance [4]. In 300 patients with TG levels exceeding 11.3 mmol/L (1000 mg/dL), excessive alcohol was present in almost a quarter of all patients and in even 43% of the highest quartile of TG levels. Especially, patients with the combination of alcohol abuse, diabetes mellitus, and obesity, for which the authors introduced the term SHIBA syndrome (severe hypertriglyceridemia influenced by alcohol), are prone to develop extremely high TG levels. In those cases, there is an increased risk of developing pancreatitis. Both the effects of excessive alcohol intake and the lack of insulin or insulin resistance push TG metabolism in the same direction.

5. Conclusions

The consumption of alcohol-containing drinks has become an accepted part of lifestyle in most societies. The health effect of alcohol, however, has always been subjected to debate. Moderate alcohol consumption is associated with a lower risk of cardiovascular disorders, and the pattern and amount of alcohol are of more importance than the type of alcoholic beverage [2, 30]. One of the underlying mechanisms for this beneficial effect is its influence on lipids especially the increase in plasma HDL-cholesterol [31]. In case of moderate drinking, 1–3 glasses a day for men and 1-2 glasses for women, hardly any effect is seen on triglycerides.

However, excessive alcohol intake may cause hypertriglyceridemia not only postprandially, but also in the fasting state. This is mainly due to an increase in the synthesis of large VLDL particles in the liver. When alcohol consumption is accompanied by a meal containing fat, especially saturated fat, it has a significant additive effect on the postprandial triglyceride peak. This peak is for the most part the result of a retardation of chylomicron breakdown and to some extent of that of VLDL remnants. Most likely, this should be attributed to an inhibition of lipoprotein lipase activity by alcohol. In case of moderate and regular alcohol intake, adaptation restores LPL activity.

In some cases, alcohol may be responsible for extremely high levels of triglycerides with an increased risk of pancreatitis. Especially, patients with the metabolic syndrome seem prone to develop such a severe hypertriglyceridemia.

Whether these changes in both postprandial and fasting triglycerides have clinical implications for cardiovascular disorders needs further exploration.

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Clinical Study

AT1 Receptor Gene Polymorphisms in relation to Postprandial Lipemia

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Background. Recent data suggest that the renin-angiotensin system may be involved in triglyceride (TG) metabolism. We explored the effect of the common A1166C and C573T polymorphisms of the angiotensin II type 1 receptor (AT1R) gene on postprandial lipemia. *Methods*. Eighty-two subjects measured daytime capillary TG, and postprandial lipemia was estimated as incremental area under the TG curve. The C573T and A1166C polymorphisms of the AT1R gene were determined. *Results*. Postprandial lipemia was significantly higher in homozygous carriers of the 1166-C allele (9.39 ± 8.36 mM*h/L) compared to homozygous carriers of the 1166-A allele (2.02 ± 6.20 mM*h/L) ($P < 0.05$). Postprandial lipemia was similar for the different C573T polymorphisms. *Conclusion*. The 1166-C allele of the AT1R gene seems to be associated with increased postprandial lipemia. These data confirm the earlier described relationships between the renin-angiotensin axis and triglyceride metabolism.

1. Introduction

Hypertriglyceridemia is an independent risk factor for the development of cardiovascular disease (CAD) [1, 2]. Recent prospective studies have shown that nonfasting TG levels are also associated with an increased risk in cardiovascular disease [3] and are possibly an even stronger predictor for CAD than fasting TG considering that humans are mostly in the nonfasting state [4, 5]. There is increasing interest to identify genes involved in the regulation of postprandial lipemia. The classical genes influencing TG metabolism like lipoprotein lipase or the APOE receptor are well known, but several others have been identified recently [6–8]. The large individual variability of TG levels cannot be fully explained by effects of these genes only. Most likely, environmental and dietary effects are the most important determinants of TG levels. However, unexpected effects by non-lipid-related genes have also been described. The renin angiotensin system mutational polymorphisms has been related to the metabolic syndrome and consequently to hypertriglyceridemia [9]. In the last decade, the beneficial effect of blockade of the renin-angiotensin system have been demonstrated in a wide variety of cardiovascular diseases, from heart failure to stable coronary artery disease and diabetic as well as nondiabetic chronic nephropathies [10, 11]. Therefore, the aim of the present study was to explore possible relationships between the angiotensin II type 1 receptor (AT1R) polymorphisms, A1166C and C573T, and fasting and postprandial triglyceridemia.

2. Material and Methods

2.1. Subjects. This paper is part of an ongoing study aimed at evaluating inheritable risk factors for premature atherosclerosis, metabolic disturbances, and genetic determinants. Normocholesterolemic CAD patients and their first-degree relatives were asked to participate. All CAD index patients had coronary sclerosis established by coronary angiography at a young age (before the age of 50 in men

and of 60 in women) and had undergone a percutaneous transluminal coronary angioplasty (PTCA) at the Heart Lung Centre Utrecht. Exclusion criteria for index patients were the presence of diabetes mellitus, body mass index (BMI) >30 kg/m^2, renal and/or liver failure, fasting plasma cholesterol >6.5 mmol/L (without lipid lowering medication), the presence of the apo E2/E2 genotype, the use of alcohol of more than 3 units a day, and a cardiac event or revascularization procedure during six months before the start of the study. Lipid-lowering medication, which was used by five patients, was stopped for 5 weeks before entering the study.

Only families of which at least two first-degree relatives were available for analysis were included. Exclusion criteria for family members were similar to those for the CAD index patients, except they should not have a medical history of CAD. Information about subjects' personal and family histories of cardiovascular disease was obtained by a standardised questionnaire. All participants were invited for a screening visit at the hospital. On the morning of inclusion blood pressure, weight, length, and waist and hip circumference were measured and a fasting blood sample was obtained for baseline determinations. The study protocol was approved by the Human Investigation Review Committees of the University Medical Centre Utrecht, and written informed consent was obtained from all participants.

2.2. TG Self-Measurements. Self-measurement of capillary TG was performed with a TG-specific point-of-care testing device (Accutrend GCT, Roche Diagnostics, Germany) [12–14]. Subjects were instructed to wash and dry their hands thoroughly before each measurement. With a lancing device, a drop of blood (30 μL) from the finger was obtained which was applied to the TG test strip in the TG analyser. Subsequently, TG concentrations were measured by a process of dry chemistry and colorimetry. Participants measured their capillary TG on three different days at six standardized time points: fasting, before and three hours after lunch and dinner, and at bedtime. The results were recorded in a diary. Subjects were requested to refrain from heavy physical activity on the measurement days. Participants did not receive recommendations concerning the frequency and composition of the meals and were requested to use their regular diet during the study. In case of one or more missing measurements during a day, the data for that particular day were not used for construction of an average diurnal TG profile. The mean diurnal TG profile of 2 or 3 days was used for statistical analysis.

The measurement range of the Accutrend GCT for capillary TG is 0.80 to 6.86 mmol/L. The Accutrend system detects TG reliably, regardless of the nature of the triglyceride-carrying lipoprotein species (chylomicrons or VLDL particles) [15]. Variation coefficients for different capillary TG concentrations are 3.3% for high TG (6.12 mmol/L) and 5.3% for low TG (1.81 mmol/L) [15]. The correlation coefficient between Accutrend capillary TG measurements and plasma measurements according to enzymatic methods is 0.94 [15]. Furthermore, in healthy lean subjects diurnal capillary TG profiles correlate to postprandial lipemia assessed by standardised oral fat-loading tests [13]. In addition, diurnal triglyceridemia estimated with 6 measurements over the day was not different compared to hourly measurements, suggesting that the chosen time points are representative for the daylong study period [14].

2.3. Analytical Methods. Blood was collected at inclusion after a 12 hours fast for measurement of plasma lipids, apolipoproteins, insulin, and glucose. Cholesterol, plasma triglyceride, and HDL cholesterol (obtained after precipitation with dextran sulphate/$MgCl_2$) were determined using a Vitros 250 analyser (Johnson & Johnson Rochester, NY, USA). Plasma apo B was measured by nephelometry using apo B monoclonal antibodies (Behring Diagnostics NV, OSAN 14/15). Plasma apo AI was measured by nephelometry using apo AI monoclonal antibodies (Behring Diagnostics NV, OUED 14/15). Plasma glucose was measured by glucose oxidase dry chemistry (Vitros GLU slides) and colorimetry and insulin was measured by competitive radio immunoassay with polyclonal antibodies. The HOMA-IR index (= glucose (mmol/L) *insulin (mU/L)/22.5) was calculated for estimation of insulin sensitivity.

2.4. Genotyping Procedures. Genotypes were determined as described previously [16]. A blood sample for polymorphism analysis was obtained from each patient in the morning after a minimum of 8 h fasting. Genomic DNA was extracted from white blood cells using silica gel polymer [17]. Reactions were conducted using DNA amplification in a final volume of 15 μL containing 0.75 μmol/L of each primer, 75 μmol/L of each NTP, 2 ng/μL DNA, 1.5 mmol/L $MgCl_2$, 75 mmol/L Tris-HCl (pH 9.0), 20 mmol/L $(NH_4)_2SO_4$, 5 mmol/L KCl, and 0.2 U/μL Netzyme DNA polymerase (Need, SL, Valencia, Spain). The DNA was amplified for 40 cycles with denaturation at 94°C for 90 s (PTC-100 thermal cycler, MI Research). The polymerase chain reaction (PCR) products underwent electrophoresis using 2% agarose gel. DNA was visualized with ethidium bromide staining. The region of the *AT1R* located between nucleotides 423 and 1278 of the cDNA was amplified using oligonucleotides 5′-GGC TTT GCT TTG TCT TGT TG and 5′-AAT GCT TGT AGC CAA AGT CAC CT as sense and antisense primers, respectively. Amplification was conducted as described above. The A1166C and C573T polymorphisms of the *AT1R* gene were analyzed simultaneously by PCR using the technique which is described in detail elsewhere [18].

2.5. Statistical Analysis. Values are given as mean ± standard deviation (SD). Comparisons between CAD index patients and healthy relatives were performed with Student's *t*-test. Comparisons between the different genotypes of the polymorphisms were performed with one-way ANOVA with Bonferroni correction for parametric data and Pearson's Chi-square for nonparametric data. In the case of TG, insulin, and HOMA index, calculations were performed after logarithmic transformation; however, untransformed concentrations are shown in the text, tables, and figures. Calculations of

TG-AUCs were performed with GraphPad Prism version 3.0 for Windows (GraphPad Software, San Diego, Calif, USA) using nonlogarithmic transformed TG concentrations. Postprandial lipemia was defined as the incremental area under the capillary TG curve after correction for fasting capillary TG (dTG-AUC). For statistical analysis, PASW version 18.0 was used. Statistical significance was reached when $P < 0.05$ (two sided).

3. Results

A total of 82 subjects were included. Sixteen of them were normocholesterolemic CAD patients, and there were 66 healthy relatives. The frequency of the T-allele for the C573T gene polymorphisms was 65%. The C-allele for the A1166C gene polymorphisms showed a frequency of 60%. No significant differences in allele distribution between patients and family members were found. All polymorphisms were in Hardy–Weinberg equilibrium. Baseline characteristics for the A1166C and C573T polymorphisms are shown in Tables 1 and 2, respectively. No significant differences were found among the different A1166C polymorphisms, but the BMI was significantly different among the C573T polymorphisms ($P = 0.047$).

Postprandial lipemia expressed as the dTG-AUC was significantly higher in homozygous carriers of the 1166-C allele (9.39 ± 8.36 mM*h/L) compared to homozygous carriers of the A-allele (2.02 ± 6.20 mM*h/L) ($P < 0.05$). Postprandial lipemia in carriers of the CA polymorphism of the A1166C gene was 3.65 ± 7.42 mM*h/L, which was intermediate to the CC and AA polymorphisms, without reaching statistical significance (Figure 1). Postprandial lipemia was similar for the different C573T gene polymorphisms ranging from 4.05 ± 9.54 mM*h/L for the CC polymorphism, 3.82 ± 4.75 mM*h/L for the CT polymorphism, to 2.17 ± 7.00 mM*h/L for the TT polymorphism (Figure 2).

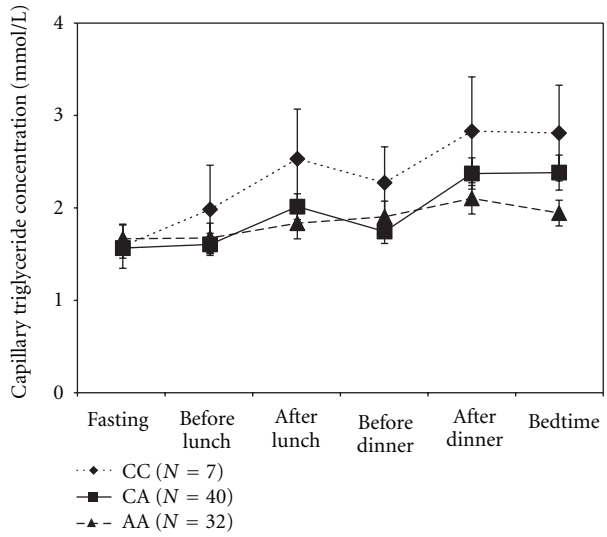

Figure 1: Diurnal capillary triglyceride (cTG) patterns for A1166C polymorphisms. Fasting cTG was similar for the three different polymorphisms, but homozygous carriers of the 1166-C allele showed a significantly increased incremental area under the capillary triglyceride curve compared to homozygous carriers of the 1166-A allele ($P < 0.05$).

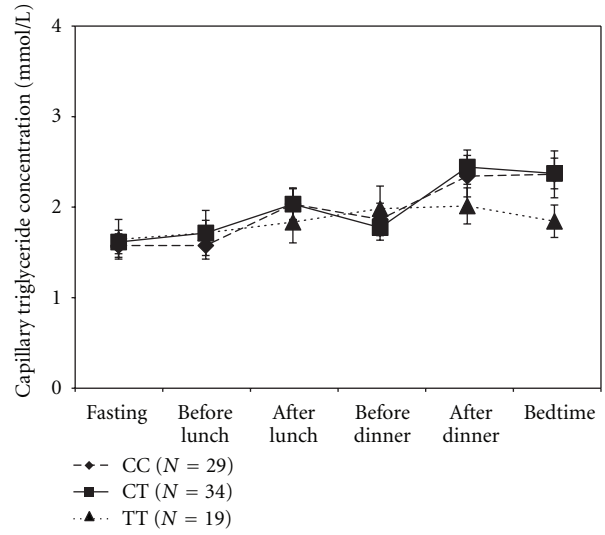

Figure 2: Diurnal capillary triglyceride patterns for C573T polymorphisms. No significant differences for the polymorphisms were found in the incremental area under the capillary triglyceride curve as a marker for postprandial lipemia.

4. Discussion

It has been suggested that 40% of the variation in triglyceride concentrations in the population is caused by genetic heritability [19]. This study shows for the first time increased postprandial lipemia in homozygous carriers of the 1166-C allele of the AT1R gene. It should be noted that the number of subjects in this study was small and only seven subjects were homozygous carriers of the 1166-C-allele. Despite the fact that fasting TG, the strongest determinant of postprandial lipemia, was similar for the groups, the CC-group showed an exaggerated postprandial response. Disturbances of lipid metabolism are frequent in patients with hypertension, metabolic syndrome, and cardiovascular disease; together they share numerous susceptibility genes [6]. Two experimental studies demonstrated increased contraction of human arteries in homozygous carriers of the 1166-C allele [20, 21]. In hypertensive patients with metabolic syndrome, the presence of the CC1166 genotype was a risk factor for central obesity and dyslipidemia [9]. Furthermore, the C-allele of the A1166C polymorphism has been associated with a decreased endothelial response to statin treatment measured with brachial artery flow-mediated dilation [22]. The C-allele also predisposes to an increased risk for stroke, especially in combination with hypertension [23]. It is known that endothelial function becomes impaired by increased TG concentrations after an oral fat-loading test [24, 25]. All these data suggest that the A1166C polymorphism of the AT1R gene shares

Table 1: Baseline characteristics for A1166C polymorphisms. Data are given as mean ± standard deviation unless stated otherwise.

	AA ($N = 34$)	CA ($N = 41$)	CC ($N = 7$)	P-value
Age (years)	43.8 ± 16.5	41.3 ± 11.0	50.0 ± 12.1	NS
Male gender	18 (53%)	22 (54%)	3 (43%)	NS
CAD	9 (26%)	5 (12%)	2 (29%)	NS
BMI	25.1 ± 3.4	25.6 ± 4.7	26.5 ± 2.9	NS
Waist (cm)	91.8 ± 11.3	92.0 ± 15.6	96.0 ± 11.8	NS
Systolic RR (mmHg)	126.6 ± 10.6	124.2 ± 14.5	127.9 ± 10.4	NS
Diastolic RR (mmHg)	82.8 ± 9.2	82.1 ± 8.1	84.3 ± 6.1	NS
Total cholesterol (mmol/L)	5.5 ± 0.9	5.3 ± 1.0	5.6 ± 0.6	NS
LDL-C (mmol/L)	3.7 ± 0.8	3.5 ± 0.9	3.6 ± 0.7	NS
HDL-C (mmol/L)	1.26 ± 0.33	1.18 ± 0.30	1.35 ± 0.40	NS
Plasma TG (mmol/L)	1.34 ± 0.62	1.37 ± 0.70	1.29 ± 0.79	NS
ApoB (g/L)	0.99 ± 0.23	0.93 ± 0.23	0.94 ± 0.20	NS
ApoA-I (g/L)	1.37 ± 0.19	1.33 ± 0.23	1.30 ± 0.28	NS
HOMA-IR	2.31 ± 1.41	2.51 ± 1.96	2.70 ± 1.75	NS

CAD: coronary artery disease, BMI: body mass index, RR: blood pressure, TG: triglyceride, Apo: apolipoprotein, HOMA-IR: homeostatic model assessment.

Table 2: Baseline characteristics for C573T polymorphisms. Data are given as mean ± standard deviation unless stated otherwise.

	CC ($N = 29$)	CT ($N = 34$)	TT ($N = 19$)	P-value
Age (years)	45.0 ± 10.4	40.7 ± 15.4	44.3 ± 15.0	NS
Male gender	13 (44.8%)	20 (58.8%)	10 (52.6%)	NS
CAD	6 (20.7%)	4 (14.7%)	5 (26.3%)	NS
BMI	26.6 ± 5.0	25.5 ± 3.6	23.7 ± 2.5	$P = 0.047$
Waist (cm)	95.2 ± 15.7	92.0 ± 12.8	88.2 ± 10.5	NS
Systolic RR (mmHg)	125.6 ± 10.9	125.3 ± 14.9	125.8 ± 11.2	NS
Diastolic RR (mmHg)	84.0 ± 7.5	80.6 ± 8.0	84.2 ± 9.8	NS
Total cholesterol (mmol/L)	5.6 ± 1.0	5.2 ± 0.8	5.4 ± 1.0	NS
LDL-C (mmol/L)	3.8 ± 1.0	3.4 ± 0.8	3.5 ± 0.9	NS
HDL-C (mmol/L)	1.29 ± 0.34	1.17 ± 0.29	1.24 ± 0.34	NS
Plasma TG (mmol/L)	1.27 ± 0.55	1.43 ± 0.73	1.33 ± 0.73	NS
ApoB (g/L)	0.97 ± 0.22	0.95 ± 0.22	0.94 ± 0.26	NS
ApoA-I (g/L)	1.35 ± 0.21	1.34 ± 0.23	1.35 ± 0.20	NS
HOMA-IR	2.77 ± 2.34	2.45 ± 1.40	2.45 ± 1.72	NS

CAD: coronary artery disease, BMI: body mass index, RR: blood pressure, TG: triglyceride, Apo: apolipoprotein, HOMA-IR: homeostatic model assessment.

susceptibility with common disorders seen in metabolic syndrome like postprandial lipemia.

The C573T polymorphism did not show any differences in postprandial lipemia. A possible explanation is that it does not influence the amino acid sequence of the encoded protein although this polymorphism is located in the coding region of the gene [21]. In contrast, the A1166C polymorphism is located in a nontranslated region of the AT1R gene but it has been suggested to be in linkage disequilibrium with another, yet-unknown, functional mutation thus explaining the multiple associations of this polymorphism with several diseases. However, contradictory results have also been found which could not confirm the association of the A1166C polymorphism with metabolic syndrome, hypertension, or cardiovascular disease [26, 27]. In one study the A1166C polymorphism was not related to triglyceride concentrations, but this study was performed in a Hong Kong Chinese population and only two subjects with the CC genotype could be identified [28]. A very recent study comprising more than 100,000 individuals did not find an association between the A1166C polymorphism and blood lipids, including TG [8]. However, the authors did not study specifically postprandial lipemia.

5. Conclusions

Although contribution of the different genes seems small, there is evidence that genetic variations have a cumulative effect on the cardiovascular risk [18, 29, 30] and that genetic variations modulate the effect of environmental factors on cardiovascular risk [31]. With the availability of easy and quick gene mapping, it might be possible in the future to genetically classify patients according to their risk,

before the disease phenotype is manifest. This might lead to preventive treatment of patients in the high-risk classes, thus diminishing disease burden and preventing unnecessary treatment of low-risk patients. In conclusion, we report that postprandial lipemia is increased in homozygous carriers of the C-1166 polymorphism of the AT1R gene. No association was found between postprandial lipemia and the C573T polymorphism.

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Review Article

The Role of Bile Acid Excretion in Atherosclerotic Coronary Artery Disease

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The impact of cholesterol and different classes of lipoproteins on the development of coronary artery disease (CAD) has been investigated in extensively during the past 50 years. The cholesterol metabolism is dependent on numerous factors, including dietary fat, fractional absorption of dietary cholesterol, tissue stores of cholesterol, endogenous cholesterol synthesis, and fecal bile excretion. Several studies showed significantly lower amounts of bile acid secretion in adult patients with CAD compared to non-CAD patients. Could it be that the inability to efficiently excrete bile acids may lead to CAD development?

1. Introduction

While a great deal of attention has been given to the factors that determine cholesterol homeostasis, cholesterol excretion via bile in patients with CAD has not been thoroughly examined [1–3].

Clinically, it became obvious that, despite effective cholesterol-modulating treatment (e.g., statins), the development of atherosclerosis cannot be stopped in a significant number of patients. It is known that cholesterol is mainly eliminated from the body via the liver in the form of bile acids [2, 4, 5]. Therefore, in addition to statins, low-density lipoprotein cholesterol (LDL-c) can be reduced by increasing the fecal bile acid waste and by compensatory hepatic upregulation of bile acid synthesis [2, 4].

It is reasonable to speculate that a reduced ability to convert cholesterol to bile acids would lead to body cholesterol overload, with the subsequent development of atherosclerosis [6–10]. The aim of this paper is to emphasize the effect of bile acid disposal on coronary artery atherosclerosis.

2. Bile Acid Excretion and Its Relationship to Coronary Atherosclerosis

2.1. Animal Studies. Studies in animals have revealed that rodents do not develop experimental atherosclerosis, despite ingestion of a cholesterol-rich diet [2, 11–13]. They were able to react to the overload of cholesterol intake by excreting large amounts of bile acids. The same results were obtained in a study on New Zealand white rabbits and primates which were also fed a cholesterol-rich diet [14]. Animals that excreted large amounts of cholesterol did not develop hypercholesterolemia, whereas those with a less efficient excretion had increased plasma level of cholesterol [14]. Furthermore, the degree of hypercholesterolemia was inversely correlated to the rate of bile acid elimination. These animal experiments suggest that the atherogenic effect of the cholesterol-rich diet closely depends on the animal's ability to eliminate cholesterol in the form of bile acids [12, 13]. It is therefore reasonable to speculate that reduced ability to convert cholesterol to bile acids would lead to cholesterol overload, with the possibility of subsequent enhanced development of atherosclerosis [6–10]. A similar correlation between the elimination of cholesterol in bile and the development of atherosclerosis was suggested for humans.

2.2. Human Studies. In an earlier investigation the elimination of bile acid in the feces of patients who had ischemic heart disease was compared with that of healthy controls on the same diet [15]. It was reported that the patients excreted much fewer bile acids than the controls [15].

Several new studies had shown an inverse relationship between CAD and bile acid excretion [6–10, 16, 17]. There are few human studies dealing with disturbed metabolism of plant sterols (which reflects cholesterol) in postmenopausal CAD women who showed disturbed synthesis and disturbed secretion of bile acids [15, 16].

These findings support the hypothesis that CAD patients produce fewer bile acids than individuals without CAD and that reduced production of bile acids could lead to advanced atherosclerosis. These findings are in line with those of the several human studies that showed increased fecal excretion of bile acids to have protective effect on CAD development [6–10, 15–17]. Most of those investigations were done on selected populations using the method described by Grundy et al. [18] for determination of total bile acids. For example, Simonen and Miettinen [19] showed that males with heterozygous familial hypercholesterolemia (FH) and CAD excreted less bile acids than control males with FH and normal coronaries. Rajaratnam et al. showed that postmenopausal women with CAD had inefficient fecal elimination of cholesterol [17].

We recently published a study on a general adult population with and without CAD and found that CAD patients eliminated subnormal amounts of fecal bile acids [3].

CAD patients excreted 358 ± 156 mg of total bile acids in comparison to healthy patients 617 ± 293 mg; $P < 0.01$. The differences in excretion were mainly due to lower excretion of deoxycholic (188.29 ± 98.12 mg versus 325.96 ± 198.57 mg; $P < 0.0001$) and less lithocholic acid (115.43 ± 71.89 mg versus 197.27 ± 126.87 mg; $P < 0.01$). Findings of this study based on 36 CAD patients and 37 non-CAD patients with a follow-up period of up to 13 years supported earlier ones and allowed for the reaching of more firm conclusions on the role of the elimination of fecal bile acids in CAD development [3].

3. Relationship between Plasma Triglycerides, HDL-Cholesterol, and Bile Acids

HDL-c has been proposed to serve as preferential precursor for bile acid biosynthesis in the liver. Furthermore, a negative relationship between plasma levels of HDL cholesterol and biliary saturation with cholesterol has been reported in healthy females [20].

In contrast to total cholesterol, LDL-c and HDL-c, there was a correlation between plasma triglycerides and bile acid excretion, but only in the non-CAD group [3]. This can be explained by a rapid and more complete intestinal absorption of triglycerides due to an excess of bile acids which are necessary for the emulsification of fats. CAD patients did not exhibit this effect because the amount of excreted bile acids was significantly lower [3].

4. Stroke and Bile Acids Excretion

Atherosclerosis is a disease characterized by lipid accumulation in the vascular wall leading to myocardial infarction or stroke [20–22]. In spite of proven efficacy of the existing drugs, like statins, cardiovascular diseases still remain the most important causes of morbidity and mortality in industrialized countries. A cholesterol-lowering effect can be achieved by reducing cholesterol synthesis or by increasing fecal excretion of bile acids (ileal sodium-dependent bile acid transporter inhibitors). It is important to emphasize that the ability to excrete large amounts of bile acids not only prevents CAD development but also may also prevent atherosclerosis in the cerebral arteries as well [3, 21, 22].

The results of the recent study after a long followup pointed out to a 6-fold higher incidence of ischemic stroke among the CAD patients compared to the non-CAD patients, 7 patients (19%) versus 1 patient (2.7%), and threefold greater mortality in the CAD group, 9 patients (25%) versus 3 patient (8%) [3].

5. 7-α Hydroxylase: The Main Enzyme Responsible for Bile Acids Excretion

The 7-α hydroxylase is the key enzyme in the conversion of cholesterol to bile acids [12, 13, 21, 23, 24]. It mediates the elimination of cholesterol from the plasma and the intracellular compartment, and it facilitates the excretion of bile acids. Thus, enhanced excretion of bile acids in non-CAD individuals can possibly be explained mainly by increased activity and concentration of 7-α-hydroxylase [12, 13, 21, 23, 24]. In contrast to it, CAD patients are unable to effectively increase the activity and concentration of 7-α-hydroxylase [23].

6. Future Perspective: Combined Treatment with Statins and Bile Acid Sequestrants

A significant percentage of patients develop atherosclerosis despite statin treatment and suppressed levels of cholesterol. We reason that, by decreasing cholesterol synthesis and increasing the utility of cholesterol, an additive antiatherosclerosis effect might be achieved. This is particularly true in the case of patients at high risk for CAD. To date it is established that these patients require aggressive lipid-lowering therapy. By combining a statin with drugs affecting bile acid and cholesterol absorption an optimal management of dyslipidemia may potentially be ensured. Additional studies are necessary to validate our contention.

7. Conclusion

Reviewing of several human studies revealed significantly lower excretion of bile acid in adult patients with CAD compared to non-CAD individuals. The diminished excretion of bile acids might be an independent risk factor for CAD and a potential target for cholesterol lowering treatment.

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Research Article

Dual AAV/IL-10 Plus STAT3 Anti-Inflammatory Gene Delivery Lowers Atherosclerosis in LDLR KO Mice, but without Increased Benefit

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Both IL-10 and STAT3 are in the same signal transduction pathway, with IL-10-bound IL10 receptor (R) acting through STAT3 for anti-inflammatory effect. To investigate possible therapeutic synergism, we delivered both full-length wild-type human (h) STAT3 and hIL-10 genes by separate adenoassociated virus type 8 (AAV8) tail vein injection into LDLR KO on HCD. Compared to control Neo gene-treated animals, individual hSTAT3 and hIL-10 delivery resulted in significant reduction in atherogenesis, as determined by larger aortic lumen size, thinner aortic wall thickness, and lower blood velocity (all statistically significant). However, dual hSTAT3/hIL-10 delivery offered no improvement in therapeutic effect. Plasma cholesterol levels in dual hSTAT3/hIL-10-treated animals were statistically higher compared to hIL-10 alone. While no advantage was seen in this case, we consider that the dual gene approach has intrinsic merit, but properly chosen partnered genes must be used.

1. Introduction

Animal and humans studies have lead to at least a partially understanding of the role of inflammatory cells in atherogenesis [1]. From this understanding we now know that atherosclerotic plaque is essentially a benign immune/inflammatory cell tumor. A cascade of events is believed to result in the development of such plaque. Endothelial cells become activated by shear stress or other insults. These activated endothelial cells then stimulate trafficking of immune cells into the intimal area, most notably monocytes. Monocytes are the precursors of macrophages and, ultimately, of lipid engorged macrophages, known as foam cells. These foam cells ultimately form the major mass of early intermediate atherosclerotic plaque. Inflammatory cytokines are overexpressed in this environment, changing the arterial lumen milieu from an antithrombogenic state to a prothrombotic one. In this altered milieu activated endothelium displays decreased nitric oxide synthesis and an increased oxidative state, which, in turn, increases inflammation [2, 3].

There are now a variety of anti-inflammatory genes already identified which might be used with therapeutic effect. Cytokine interleukin 10 (IL-10) has been significantly studied as a strong general inhibitor of immune response and inflammation. In mouse models IL-10 gene delivery does result in moderate antiatherosclerotic effects, shown by multiple groups [4–6]. It is also known that IL-10 signaling goes through, requires, signal transducer and activator of transcription 3 (STAT3) [7, 8] and that STAT3 gene delivery also results in inhibition of atherosclerotic plaque formation [9]. Yet, while individual IL-10 and STAT3 gene delivery were efficacious, some disease remained. Thus, yet stronger gene effectors are desirable. This issue is particularly important considering that the most likely candidates for antiatherosclerosis gene therapy will have significant established disease.

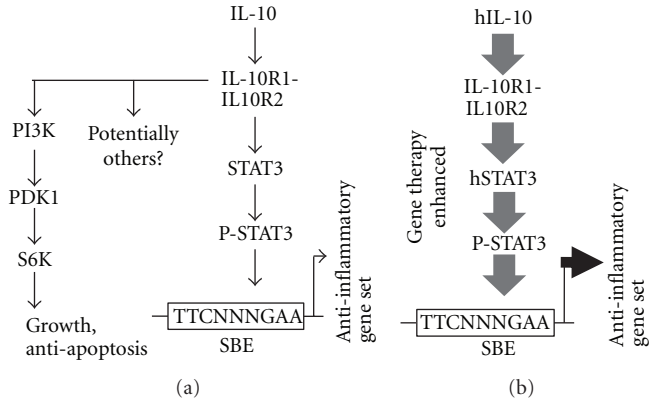

Figure 1: IL-10 signal transduction and hypothesized treatment by dual gene therapy. (a) shows multiple possible IL-10 signal transduction pathways. The STAT3 pathway results in an anti-inflammatory response. (b) shows our attempt to enhance IL-10 signaling at two points with the hypothesis that enhanced gene expression of the anti-inflammatory gene set would occur. The gene delivery is indicated by larger font and bolded IL-10 and STAT3. More powerful signal transduction is represented by larger arrows and larger font.

In the field of cancer it is well known that multiple protooncogene mutations often take place within a single signal transduction pathway, resulting in continuous and permanent signaling within those malignant cells, most commonly signaling continuous cell division [10]. This is a powerful form of functional gene cooperation which ultimately results in many cancer deaths. We considered this cancer-causing gene signaling cooperation to have a powerful phenotype and that this strategy could potentially be useful for addressing atherogenesis. We hypothesized that perhaps the overexpression of two genes within a common signal transduction pathway, using anti-inflammatory genes in place of oncogenes, may result in a beneficial gene cooperation and provide an even stronger anti-inflammatory effect. Moreover, this is a novel gene therapy approach, not attempted before.

We have previously studied the individual use of the IL-10 and STAT3 genes to lower inflammation and atherogenesis [4, 9]. These two genes are located within the same anti-inflammatory signal transduction network, with IL-10 acting through STAT3 [7, 8]. To test the hypothesis that two anti-inflammatory genes of the same pathway will have higher efficacy than one, hIL-10 plus hSTAT3 was delivered using adenoassociated virus (AAV) as a gene delivery vector [4, 9], and the resulting therapeutic effect was studied in a LDLR KO mouse-HCD model. AAV is an outstanding gene therapy/gene delivery vector, having been used since 1984 [11–13], and does not contribute to inflammation [14]. Contrary to our thoughtful planning, no increased therapeutic benefit was observed when using STAT3/IL-10 together, and, in fact, cholesterol levels were higher in the dual-gene-treated animals.

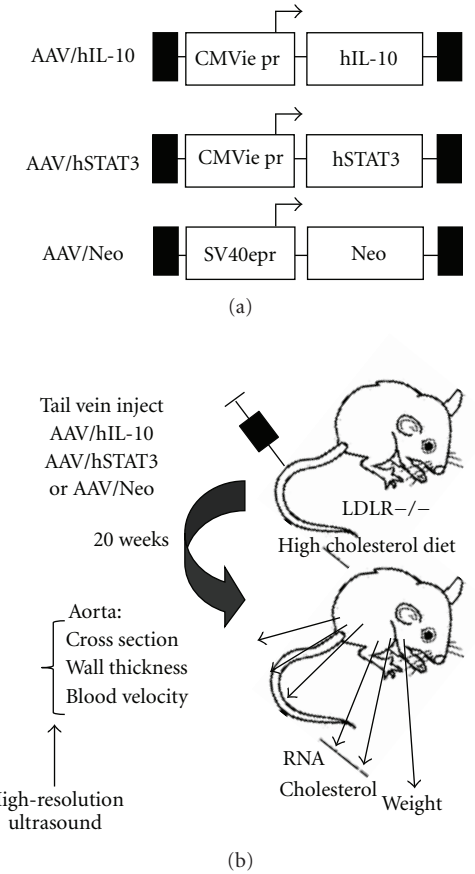

Figure 2: Structure of AAV vectors and experimental scheme. (a) shows the basic structure of the three AAV vectors used in this study. (b) shows the experimental scheme and data collected.

2. Methods

2.1. Generation of Recombinant AAV Virus. Construction and generation of AAV/Neo, AAV/hSTAT3, and AAV/hIL-10 recombinant virus have been described previously [4, 7, 9]. The virus stocks were generated and tittered by dot blot hybridization as described previously [4, 7, 9]. The titers were calculated to be about 1×10^9 encapsidated genomes per mL (eg/mL).

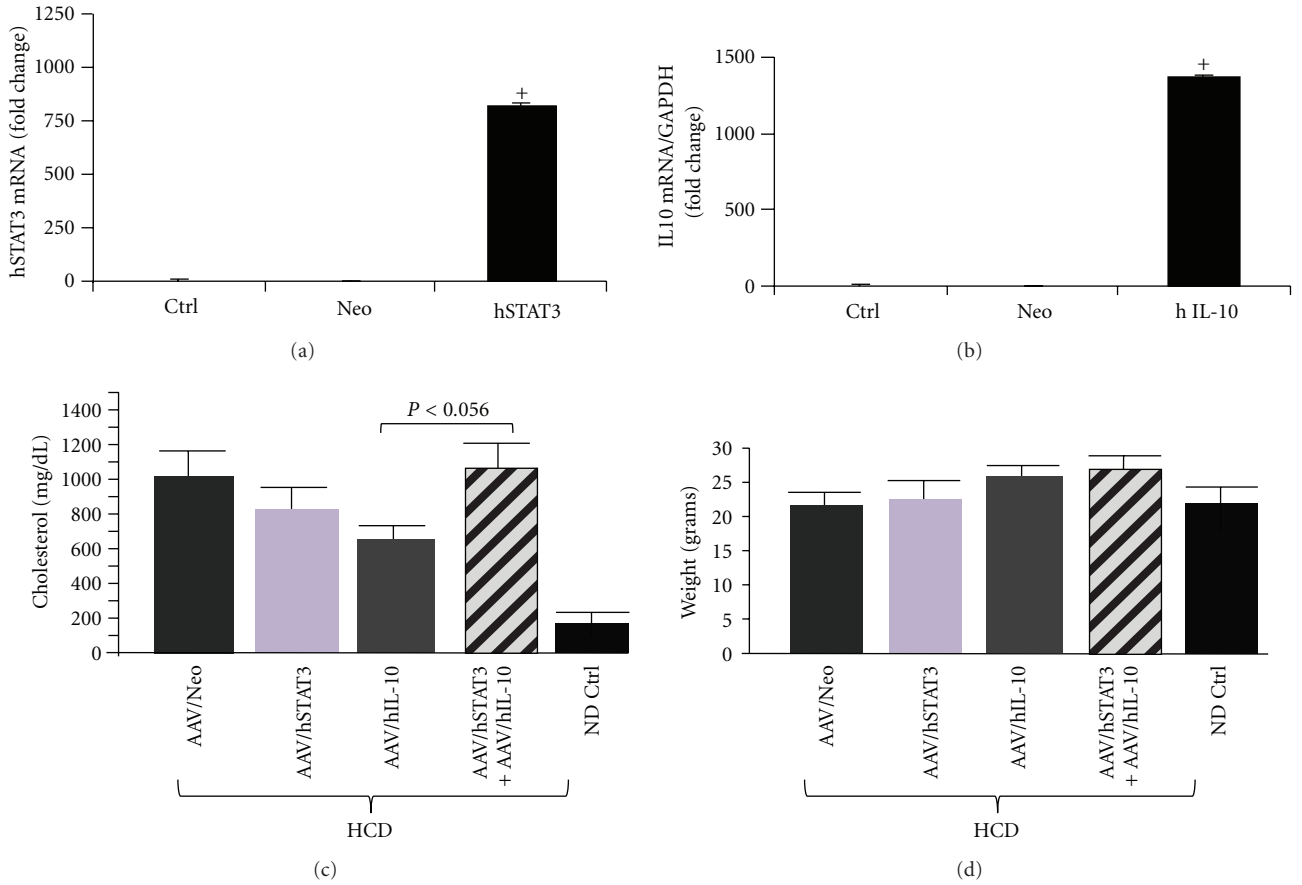

Figure 3: Expression of the delivered hSTAT3 and IL-10. Relative expression of hSTAT3 (a) and IL-10 (b) genes by real-time quantitative PCR from aorta of 3 mice in each group. For qRT-PCR the quantity of RNA for each gene was normalized to GAPDH in the same sample. Data shown are mean ± SE. (c) shows the levels of total cholesterol. Note that cholesterol levels of the hSTAT3-plus-hIL-10-treated animals had significantly higher cholesterol levels than animals treated with individual genes. (d) shows the animal weights at the end of the experiment. The key at the bottom is used for both panels (c) and (d).

2.2. Animal Treatments. Low-density lipoprotein receptor (LDLR) knockout mice (B6;129S7-*Ldlr*^{tm1Her}/J) were obtained from Jackson Laboratories (Bar Harbor, Me, USA). Two groups of male mice weighing 16–20 grams were injected with AAV-Neo and AAV-STAT3 virus each at a titer of 1×10^9 eg/mL via tail vein injections of 200 μl virus/mouse, followed by two booster injections at an interval of less than one week. The animals were started on high cholesterol diet (HCD) of 4% cholesterol and 10% Coco butter diet (Harlan Teklad, Madison, Wis, USA) on the day of first injection and maintained for twenty weeks. This HCD was used to ensure the development of atherosclerosis. Mice fed normal chow diet were included as experimental control group. Animals were weighed weekly, and all experimental procedures were performed in accordance with protocols approved by the Institutional Animal Care and Usage Committee of the Central Arkansas Veterans Health Care System at Little Rock.

2.3. High-Resolution Ultrasound Imaging. Ultrasound imaging was done using the Vevo 770 High-Resolution Imaging system (Visualsonics, Toronto, Canada) with a RMV 707B transducer having a center frequency of 30 MHz. Animal preparation was done as described earlier [15]. Briefly, each mouse was anesthetized using 1.5% isoflurane (Isothesia, Abbot Laboratories, Chicago, USA) with oxygen and laid supine on a thermostatically heated platform with all legs taped to ECG electrodes for cardiac function monitoring. Abdominal hair was removed with a shaver and a chemical hair remover (Church & Dwight Co., Inc., NJ, USA), and a prewarmed US gel (Medline Industries, Inc., Mundelein, USA) was spread over the skin as a coupling medium for the transducer. Two levels of the vessel were visualized: thoracic region—below the aortic arches to the diaphragm and the renal region—the upper abdominal region to the iliac bifurcation. Image acquisition was started on B-mode, where a long-axis view was used to visualize the length of the aorta. Next, the scanhead probe was turned 90° for a short-axis view to visualize the cross-sectional area of the aorta. Individual frames and cine loops (300 frames) were acquired at all levels of the aorta both in long-axis and short-axis view and recorded at distances of 1 mm throughout the length of the aorta. For measurement of flow velocity, orientation of the abdominal aorta on ultrasound was accomplished by

tilting the platform and the head of mouse down with the transducer probe towards the feet and tail of the mouse. This positioning ensured the Doppler angle to be less than 60° for accurate measurements of blood flow velocity in the pulse wave Doppler (PW) mode within abdominal aorta. Measurements and data analysis was performed off line using the customized version of Vevo 770 Analytical Software from both the longitudinal and transverse images. The complete imaging for each mouse lasted for about 25–30 minutes.

2.4. Measurement of Plasma Cholesterol. Plasma levels of total cholesterol for AAV8/hSTAT3 and AAV8/Neo mice were measured by VetScan VS2 (ABAXIS, Union City, Calif) at the Veterans Animal Laboratory (VAMU).

2.5. hSTAT3 and IL-10 Gene Expression Analysis Using Real-Time Quantitative Reverse Transcription PCR (qRT-PCR). Total RNA from aorta of three mice was extracted with TRIzol extraction (Invitrogen Carlsbad, Calif) according to the manufacturer's instructions. cDNA was synthesized using random hexamer primers and RNase H-reverse transcriptase (Invitrogen, Carlsbad, Calif). QRT-PCR was performed using the Applied Biosystems Fast 7900HT real-time PCR system (Applied Biosystems, Foster City, Calif) as described in [9]. We designed qRT-PCR specific primers for analyzing hSTAT3 and IL-10 using ProbeFinder (http://www.roche-applied-science.com) web-based software from Human and Mouse Universal ProbeLibrary from Roche Applied Science. The results were analyzed using SDS 2.3 relative quantification (RQ) manager software. The comparative threshold cycles (Ct) values were normalized for GAPDH reference genes and compared with a calibrator by the $2^{-\Delta\Delta Ct}$ method.

3. Results

3.1. AAV8 Delivers hIL-10 and hSTAT3. Both IL-10 and STAT3 have been shown to inhibit atherosclerosis in an animal model [4–6, 9], and both are in the same anti-inflammatory signal transduction pathway, with IL-10 acting through STAT3 [7, 8]. Because of this we reasoned that the delivery of both genes together may result in a synergistic higher level of anti-inflammatory activity, which is depicted in Figure 1. To test this hypothesis and the efficacy of IL-10-plus-STAT-3 dual gene delivery we delivered both into LDLR KO mice using AAV8 and placed them on high cholesterol diet (HCD). An AAV/Neomycin resistance gene (Neo) vector was also used as a null, nontherapeutic control. Vector structures are shown in Figure 2(a) and the overall experimental scheme in Figure 2(b). Upon time of harvest, a portion of mice were sacrificed to determine the success of gene delivery by analyzing hSTAT3 mRNA expression in the aorta using qRT-PCR analysis. This analysis utilized mRNA isolated from 3 mice aortas from each group, harvested at week 20. Representative results for hSTAT3 and hIL-10 are shown in Figure 3(a), and both were observed to be highly expressed in aortas of appropriately treated animals but not AAV/Neo-injected or control animals.

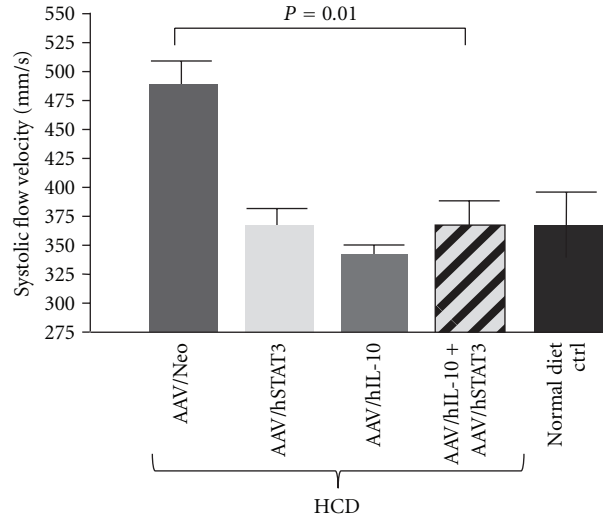

Figure 4: Systolic blood velocity. High-resolution ultrasound (HRUS) was used to measure blood flow velocities in the lumenal center of the abdominal region of the aorta in 3–5 animals from each group. Shown is a quantification of the results. Note that the hSTAT3-treated, the hIL-10-treated, and the hSTAT3-plus-hIL-10-treated animals all had significantly lower blood velocity than the AAV/Neo animals.

3.2. Therapeutic hSTAT3, hIL-10, or hSTAT3-Plus-hIL-10 Gene Delivery Inhibits Aortic Blood Flow Velocity with Equal Efficacy. Following demonstration of successful transgene delivery studied the effects of the transgene. Figure 3(b) shows that hSTAT3-treated animals had total cholesterol levels comparable to Neo-treated HCD-fed animals; however hIL-10-treated animals were statistically lower. Yet it was found that the dual hIL-10-plus-hSTAT3-treated animals had cholesterol levels which trended higher than either hIL-10- or hSTAT3-treated animals. Regarding animal weights, all treatments were statistically the same as normal diet (ND) controls but trended higher.

We utilized blood flow velocity measurement as a novel technique to quantify atherosclerosis in the mice. Systolic blood flow velocity in the lower abdominal region of the aorta was quantified by high-resolution ultrasound (HRUS) imaging system Vevo 770 with measurements taken on three to five animals. Figure 4 shows the quantified the systolic blood velocity from five separate measurements on each animal. As shown, the AAV/Neo-HCD-treated animals, with the highest lipid deposition, displayed the highest flow velocity. Moreover, all three therapeutic treatments had markedly lower flow velocity, very similar to that in normal-diet-fed control animals. Thus all three treatments showed antiatherosclerotic efficacy, yet the dual gene treatment was not statistically improved over the individual gene treatments, nor did dual gene delivery trend towards higher efficacy.

3.3. Therapeutic hSTAT3, hIL-10, and hSTAT3-Plus-hIL-10 Gene Delivery Inhibits Aortic Structural Changes Associated with Atherosclerosis with Equal Efficacy. Next structural

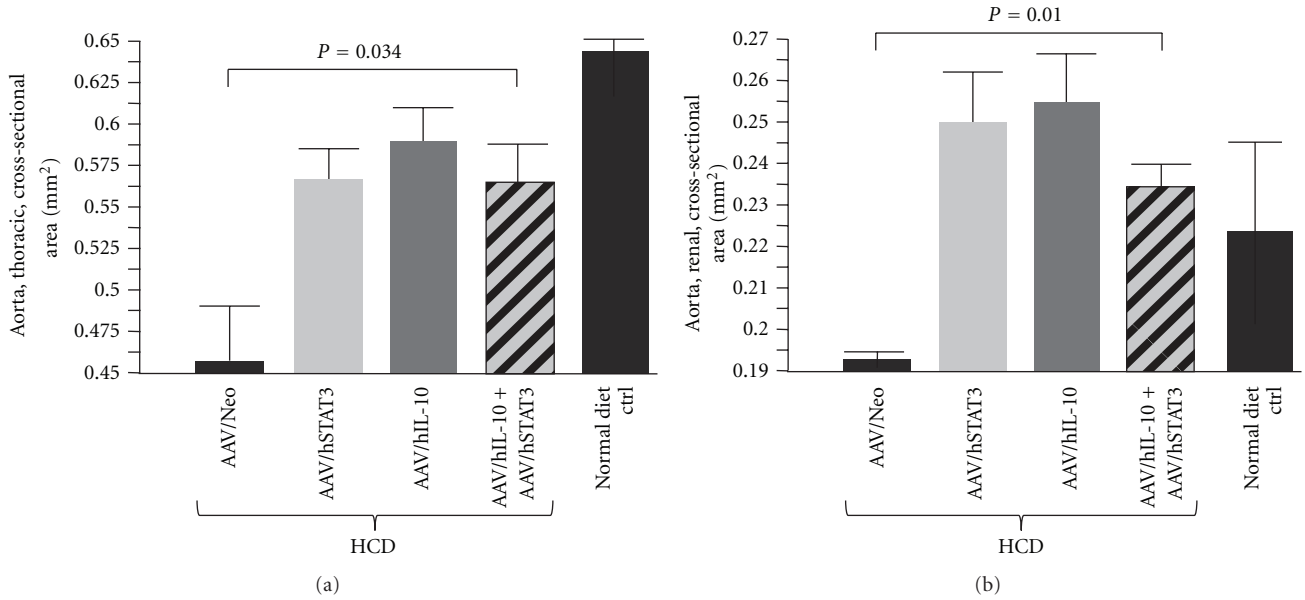

Figure 5: Analysis of the aortic lumen by high-resolution ultrasound (HRUS). HRUS was used to measure the cross-sectional area of the thoracic and renal regions of the aortas in 3–5 animals from each animal group. (a) shows quantification of the cross-sectional area for the thoracic region of the aorta. Note that the hSTAT3-treated, the hIL-10-treated, and the hSTAT3-plus-hIL-10-treated animals all had a much larger cross sectional area than the AAV/Neo-treated animals, indicating significant efficacy. Similarly in (b), quantification of the data for the renal region of the aorta shows a much larger lumen size for the hSTAT3-, hIL-10-, and hSTAT3-plus-hIL-10-treated animals compared to AAV/Neo-treated animals.

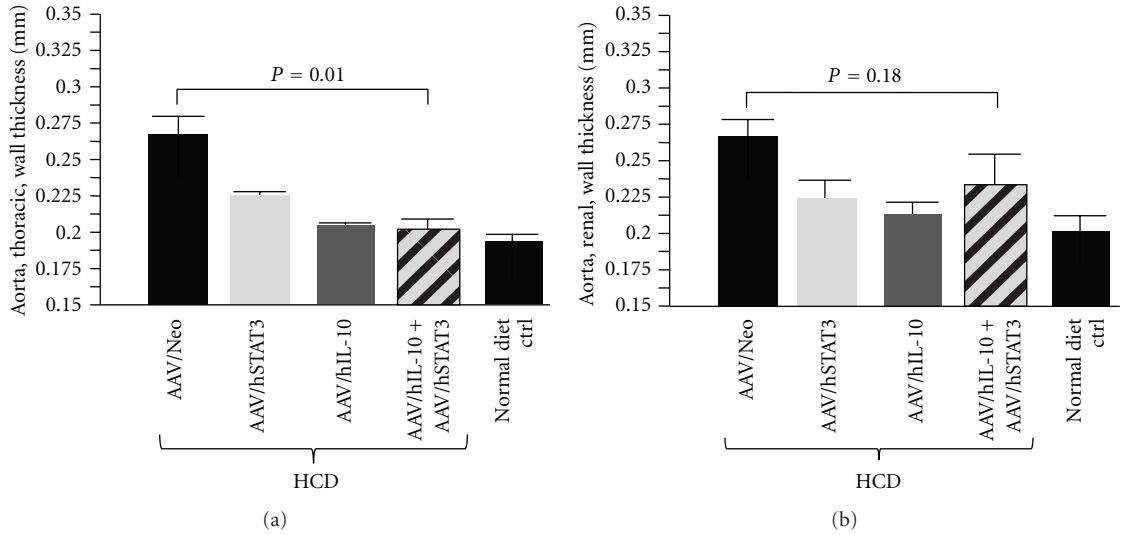

Figure 6: Analysis of the aortic wall thickness by HRUS. HRUS was used to measure the wall thickness of the aorta. (a) shows quanitification of the thoracic region of the aortas in 3–5 animals from each animal group. Note that the hSTAT3-, hIL-10-, and hSTAT3-plus-hIL-10-treated animals had thinner wall thicknesses than the Neo-treated animals, indicating significant efficacy. (b) shows quantification of the renal region of the aortas in 3–5 animals from each animal group.

changes in the aortas resulting from the various treatments were quantified using HRUS imaging. Multiple measurements were made in three to five animals from each group and compared. The measurements were made in the same indicated site (see Section 2). The cross-sectional area of the lumen of the thoracic region of the aorta was one measurement taken, with five readings from each animal. Figure 5(a) shows the quantified results for the thoracic region of the aorta. The AAV/Neo-HCD-treated positive control animals displayed the smallest cross sectional lumen area, consistent with significant atherosclerosis. In contrast, all three therapeutic treatments (hSTAT3, hIL-10 or hSTAT3-plus-hIL-10) had much larger lumens than Neo-treated-HCD controls, and this difference was statistically significant.

Normal diet control animals had the largest lumen. The renal region of the aorta showed a similar pattern to the thoracic aorta, as shown in Figure 5(b), with AAV/Neo-HCD-treated having the smallest and all three therapeutic treatments having statistically significant larger lumens. Thus, again, all three treatments showed antiatherosclerotic efficacy, yet the dual gene treatment was not statistically improved over the individual gene treatments.

The wall thickness of the thoracic region of the aorta was yet another measurement made, with data from three to five animals and five readings from each animal. Figure 6 shows the quantified results for the thoracic region of the aorta. The AAV/Neo-treated animals displayed the thickest thoracic walls, and all three therapeutic treatments gave statistically thinner walls, ($P = 0.05$). Yet, again, dual gene delivery failed to improve therapeutic efficacy.

4. Discussion

This study is the first attempt at using dual gene therapy within a single signal transduction pathway with a believed beneficial phenotype with the hypothesis that improved efficacy will result. Both IL-10 and STAT3 are anti-inflammatory genes within the same signal transduction pathway. Because of this we hypothesized that a synergistic anti-inflammatory effect or enhanced therapeutic effect might be observed by both AAV8/hIL-10 plus AAV8/hSTAT3 dual gene delivery in LDLR KO mouse on HCD. This study demonstrates that there was no enhanced efficacy when both genes were delivered together over that of the individual genes. One possible explanation for this lack of effect is that the two genes were delivered by separate AAV vectors and thus likely that very few cells were receiving and actively expressing both transgenes. If the proteins of both genes were intracellular then this explanation would likely be correct and fully prevent gene cooperation. However, while hSTAT3 is intracellular, IL-10 is a secreted protein and its activity should be effective over an area through its diffusion. Thus, we think that this explanation is not a viable mechanism as to why there is no additive or synergistic therapeutic effect by this approach. Another possible contributor to the lack of enhancement is that IL-10 trends to have a greater effect than STAT3; thus what we may be viewing is only the dominant IL-10 effect. Alternatively, the lack of improved efficacy by dual IL-10/STAT3 delivery may also suggest that another IL-10 pathway is more effective in inhibiting inflammation than the STAT3 pathway. Yet enhancement of the PI3K pathway would seem inappropriate as PI3k expression appears positively associated with atherosclerosis [16].

Cholesterol levels seen in the dual-gene-treated animals were higher than in either individual gene-treated animals (statistically significant for hIL-10 versus hIL-10-plus-hSTAT3). As both IL-10 and STAT3 are individually associated with lower cholesterol levels [5, 6, 9], these data may suggest that some type of negative regulatory interference between these two gene's functions and their downstream signaling is taking place. Additionally, their individual mechanisms for lowering cholesterol may also be abrogated. The mechanism of how each lowers blood cholesterol levels is presently unclear.

Yet another possibility for the failure of IL-10 and STAT3 to cooperate may be the "crossroads" position of STAT3 in the transduction of signaling from multiple receptors, beyond IL-10/IL-10R interaction. Other important signaling pathways which require STAT3 signaling include IL-6 [17], IL-17 [18], Ang II [19], and thrombin [20]. While it is unclear which of these pathways (or others) is active and might serve to inhibit the IL-10-STAT3 signaling pathway, this study reminds us of the complexity of cellular signaling and the need to determine signaling pathway dominance or interference when medically significant issues of IL-10 and STAT3 are at issue. An alternative gene pair which might be tried would be IL-10 plus IL10R1. This would concentrate augmentation at only the ligand-receptor level, and the complexities of STAT3 overexpression might be avoided.

It is surprising that IL-10 and STAT3 overexpression should apparently "knock out" each other's cholesterol regulation phenotype as well as limit each other's antiatherogenesis phenotype. Due to our negative results we did not investigate the failure of cooperation between IL-10 and STAT3 any further. Pursuing the mechanism of action of negative data is unjustified. However, these results of noncooperation indicate that our understanding of the IL-10 and STAT3 pathways is incomplete and that gene pairs utilized in dual gene therapies may give surprising results different than what initial logical planning might suggest. However, we believe the dual gene delivery approach will ultimately result in enhanced efficacy if the gene partners chosen are mechanistically appropriate and compatible. In spite of the negative results presented here, the investigation and discovery therapeutic gene synergisms, but not between IL-10 and STAT3, has merit and needs to be pursued.

Acknowledgments

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Review Article

Hypertriglyceridemia, Metabolic Syndrome, and Cardiovascular Disease in HIV-Infected Patients: Effects of Antiretroviral Therapy and Adipose Tissue Distribution

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The use of combination antiretroviral therapy (CART) in HIV-infected patients has resulted in a dramatic decline in AIDS-related mortality. However, mortality due to non-AIDS conditions, particularly cardiovascular disease (CVD) seems to increase in this population. CART has been associated with several metabolic risk factors, including insulin resistance, low HDL-cholesterol, hypertriglyceridemia and postprandial hyperlipidemia. In addition, HIV itself, as well as specific antiretroviral agents, may further increase cardiovascular risk by interfering with endothelial function. As the HIV population is aging, CVD may become an increasingly growing health problem in the future. Therefore, early diagnosis and treatment of cardiovascular risk factors is warranted in this population. This paper reviews the contribution of both, HIV infection and CART, to insulin resistance, postprandial hyperlipidemia and cardiovascular risk in HIV-infected patients. Strategies to reduce cardiovascular risk are also discussed.

1. Introduction

The widespread use of combination antiretroviral therapy (CART) has led to a dramatic and sustained reduction in the morbidity and mortality associated with HIV infection and has transformed this disease into a chronic condition [1, 2]. CART generally consists of two nucleoside analogue reverse-transcriptase inhibitors (NRTIs) and a protease inhibitor (PI) or nonnucleoside analogue reverse-transcriptase inhibitor (NNRTI). Despite an enormous decrease in AIDS-related mortality, CART has been associated with changes in body fat distribution and several metabolic risk factors, such as hypertriglyceridemia, low HDL-cholesterol, and insulin resistance [3–5]. Moreover, recent studies have shown that prolonged use of CART is associated with an increased risk of cardiovascular disease (CVD) [6, 7]. As treatment of HIV infection has become more successful, CVD may become an increasingly growing health problem in HIV-infected patients. This review focuses on the underlying mechanisms and characteristics of dyslipidemia, insulin resistance, and CVD in HIV-infected patients.

2. Lipodystrophy

CART in HIV-infected patients is strongly associated with changes in body fat distribution, often referred to as lipodystrophy [3–5]. Lipodystrophy is characterized by subcutaneous fat loss, visceral fat accumulation, and development of a buffalo hump. Subcutaneous fat loss is most noticeable in the face, limbs, and buttocks and may occur independently of visceral fat accumulation. The prevalence of lipodystrophy varies widely, from 10 to 80 percent, and is mainly dependent on the type and duration of CART and the criteria used for diagnosing lipodystrophy [3–5]. Severe forms of lipodystrophy, especially lipoatrophy, can be disfiguring and stigmatizing and often lead to suboptimal adherence to CART. All classes of antiretroviral agents may

be related to the development of lipodystrophy, but the prevalence and severity of lipodystrophy are increased mostly in patients treated with the combination of NRTIs and a PI [3–5]. The etiology of lipodystrophy appears to be multifactorial, including HIV drug inhibitory effects on adipocyte differentiation and alteration of mitochondrial functions. PIs impede adipocyte differentiation through altered expression and nuclear localization of sterol regulatory element-binding protein-1 (SREBP-1) and peroxisome proliferator-activated receptor-γ (PPAR-γ), which are essential for adipogenesis [8]. NRTIs may induce mitochondrial dysfunction and apoptosis of adipocytes by inhibition of mitochondrial DNA polymerase-γ and depletion of mitochondrial DNA [9].

3. Dyslipidemia

The natural course of HIV infection is characterized by reductions in HDL-cholesterol and LDL-cholesterol and an increase in triglycerides (TGs) [10]. Elevated TGs are due to a combination of hepatic very low-density lipoprotein (VLDL) overproduction and reduced TG clearance [10, 11]. Hypertriglyceridemia is related to poor virological control and increased levels of TNF-α [10, 11]. TNF-α interferes with free fatty acid (FFA) metabolism and lipid oxidation and attenuates insulin-mediated suppression of lipolysis [11]. The nutritional state of HIV-infected patients, including weight loss and protein depletion, contributes to reduced HDL-cholesterol and LDL-cholesterol levels [10, 11].

Following the introduction of CART, more pronounced atherogenic changes in the lipid profile, including increases in TG and LDL-cholesterol, and a decrease in HDL-cholesterol, have been observed [3–5]. In addition, increases in apolipoprotein B (apoB) have been found, often associated with the predominance of atherogenic small dense LDL [3–5]. In a large cross-sectional study, the prevalence of hypercholesterolemia (>6.2 mmol/L), hypertriglyceridemia (>2.3 mmol/L), and low HDL-cholesterol (<0.9 mmol/L) was 10 to 27 percent, 23 to 40 percent, and 19 to 27 percent, respectively, depending on the antiretroviral regimen [12]. The pathogenesis of CART-related dyslipidemia is complex and involves various drug-induced effects, in association with hormonal and immunological influences. Especially PI therapy has been associated with dyslipidemia. The most pronounced changes in the lipid profile have been observed with intensive booster doses of ritonavir [13]. Amprenavir and nelfinavir have intermediate effects on plasma lipids, while indinavir, saquinavir, and lopinavir have minor effects on plasma lipids [3–5, 13]. Atazanavir does not negatively affect the lipid profile [14]. The effects of PIs on the lipid profile seem to be drug-related, because an interaction with the host response to HIV or changes in body composition has been excluded by several short-term studies conducted in HIV-negative volunteers [15–17]. In these subjects, ritonavir increased the concentrations of plasma TG, apoB, and VLDL-cholesterol as early as 2 weeks after administration [15]. Administration of lopinavir/ritonavir to healthy HIV-negative volunteers for 4 weeks resulted in increased TG and decreased HDL-cholesterol levels [16]. In contrast, Noor et al. showed that administration of indinavir for 4 weeks to HIV-negative subjects did not result in significant changes in lipoproteins, TG, or FFA levels but caused insulin resistance independent of increases in visceral adipose tissue [17]. Apparently, changes in the lipid profile develop as early as weeks after administration of a PI, independent of HIV infection or body fat distribution, suggesting direct effects of PIs on lipid metabolism.

The mechanism of PI-induced dyslipidemia is not fully understood but is probably multifactorial. First, it has been suggested that PIs suppress the breakdown of the nuclear form of SREBP-1 in the liver [18]. SREBP-1 is a master transcriptional regulator and regulates the expression of genes involved in FFA, TG, and cholesterol biosynthesis. Hence, hepatic accumulation of SREBP-1 could result in increased hepatic de novo lipogenesis. For example, ritonavir has been shown to increase the level of active ADD-1/SREBP-1 protein during adipogenesis [18]. Similarly, indinavir and nelfinavir, but not amprenavir, altered adipose cell differentiation and SREBP-1 nuclear localization in an adipose cell line [19]. Second, PIs seem to suppress proteasomal breakdown of nascent apoB in the liver, leading to increased VLDL secretion [20]. In human hepatoma (HepG2) cells, treatment with therapeutically relevant concentrations of ritonavir or saquinavir protected nascent apoB from intracellular degradation [20]. Whether this is also the case for the other PIs is not known. A third proposed pathway is based upon the structural similarity between the catalytic region of HIV-1 protease and two homologous proteins involved in lipid metabolism: cytoplasmic retinoic acid-binding protein type 1 (CRABP-1) and low density lipoprotein-receptor-related protein (LRP) [4]. CRABP-1 is involved in the conversion of retinoic acid to cis-9-retinoic acid, which binds the retinoid X receptor- (RXR-)PPAR-γ heterodimer, stimulating adipocyte differentiation and proliferation. PIs are thought to bind to CRABP-1 and inhibit the formation of cis-9-retinoic acid, leading to reduced PPAR-γ activity and peripheral lipoatrophy [4]. Impaired FFA storage capacity in adipose tissue and increased flux of circulating lipids may upregulate hepatic VLDL production and, hence, contribute to hyperlipidemia. LRP normally binds to lipoprotein lipase (LPL) on capillary endothelium, which hydrolyses FFA from TG, promoting their accumulation in adipocytes. Remnants of TG-rich lipoproteins (TRLs) are removed from the circulation by the LDL-receptor and the hypothetical remnant receptor in the liver. Binding of PIs to LRP would impair hepatic remnant uptake and TG clearance by the endothelial LRP-LPL complex [4]. Evidence supporting this concept has been provided in apoE3-Leiden transgenic mice treated with ritonavir, demonstrating decreased LPL-mediated TG clearance as well as impaired uptake of TG-derived FFA in adipose tissue, which may contribute to lipodystrophy [21].

PI-sparing CART may also affect the lipid profile, although cholesterol and TG levels generally rise less in comparison with regimens containing a PI [12, 22]. Of the NNRTIs, efavirenz is associated with higher levels of cholesterol and TG than is nevirapine, whereas both increase HDL-cholesterol [23]. The severity and prevalence of dyslipidemia in HIV-infected patients may also depend on HIV disease stage and the concomitant presence of lipodystrophy and

insulin resistance [3–5, 12]. The presence of lipodystrophy in HIV-infected patients has been associated with accelerated lipolysis, hepatic reesterification, and hypertriglyceridemia [24]. However, although it is likely that increased FFA release from adipose tissue contributes to the increase in hepatic VLDL synthesis, other factors must be involved, because insulin-induced suppression of lipolysis and systemic FFA availability did not normalize the VLDL-TG secretion rate in a kinetic study [25].

4. Postprandial Lipemia

TRLs are mainly produced in the postprandial phase. A schematic overview of postprandial TG and FFA metabolism is shown in Figure 1. Endogenous TRLs (VLDL, containing apoB100 as structural protein) and exogenous TRLs (chylomicrons, containing apoB48 as structural protein) compete for clearance by LPL, which hydrolyzes TG into glycerol and FFA, leaving atherogenic remnant particles [26]. In the postprandial phase due to limited LPL availability, competition at the level of LPL will occur resulting in accumulation of TRL. This competition is most likely when fasting hypertriglyceridemia is present. The lipolytic rate, as well as the clearance of remnant particles by liver receptors, contributes to removal of TRL from the circulation. Adipose tissue plays a crucial role in regulating FFA concentrations in the postprandial period by suppressing the release of FFA in the circulation and stimulating the uptake of FFA liberated from TRL by LPL [27]. This pathway is also known as the pathway of adipocyte FFA trapping.

A schematic overview of the proposed mechanism for hypertriglyceridemia in HIV-infected patients is shown in Figure 2. First, direct effects of PIs on hepatic TG synthesis and apoB metabolism, which have been described in detail in the previous section, may result in increased VLDL secretion. Insulin resistance and the presence of lipodystrophy may further contribute to hypertriglyceridemia. Adipocyte FFA trapping is impaired in patients with HIV-lipodystrophy, because there is not sufficient subcutaneous adipose tissue to provide the necessary buffering capacity [28]. If adipocyte FFA trapping is disturbed, then nonadipose tissues, such as the liver, skeletal muscle, and pancreas, are exposed to excessive FFA concentrations. FFA reaching the liver may contribute to an increase in VLDL synthesis [28]. In addition, impaired disposal of intestinal TRL is related to a defect in LPL activity in HIV-infected patients [28–33]. Especially the combined use of NRTIs and PIs has been associated with impaired clearance of TRL [30]. In a kinetic study, it was shown that HIV-infected patients on CART show a significant reduction in VLDL and IDL apoB fractional catabolic rates compared with HIV-negative controls, which was related to peripheral fat loss [31]. Even in fasting normolipidemic subjects, CART resulted in higher postprandial remnant lipoprotein levels, irrespective of the CART regimen [33]. Increasing evidence suggests that postprandial hyperlipidemia contributes to atherosclerosis [34–37]. Both hepatic and intestinal TRL and their remnants accumulate in the subendothelial space, where they promote atherosclerosis by the formation of foam cells [34]. Hence, current evidence suggests a proatherogenic postprandial lipoprotein phenotype in CART-treated HIV-infected patients, with accumulation of remnant lipoproteins.

5. Adipose Tissue

In the general population, postprandial TG and FFA metabolism is closely related to adipose tissue distribution. Especially abdominal obesity is associated with insulin resistance and hypertriglyceridemia [38]. However, absolute or partial lack of body fat may result in a similar metabolic risk profile. Several forms of congenital and acquired lipodystrophies have been related to dyslipidemia, insulin resistance, and early onset DM [39–41]. Similarly, the presence of CART-associated lipodystrophy in HIV-infected patients may play a key role in disturbed TG metabolism.

How does the lack of body fat lead to similar manifestations to those seen with an excess of fat? There may be several explanations. First, experimental evidence indicates that subcutaneous fat may be considered a "metabolic sink" that prevents accumulation of harmful ectopic fat [39–41]. Lack of adipose tissue diverts TG and FFA to accumulate in other organs, such as the liver and skeletal muscle, leading to hepatic fat accumulation, VLDL overproduction, and insulin resistance [39–41]. In line with this hypothesis, the presence of HIV-lipoatrophy has been associated with impaired adipocyte FFA trapping [28], increased fat content in the liver [42], VLDL overproduction [24, 28], and impaired disposal of TRL [25, 29]. Most likely, the effects on dyslipidemia are resulting from lipodystrophy due to CART, although a role of HIV infection itself cannot be excluded. Second, in addition to the central role of lipid storage, adipose tissue also releases a large number of cytokines and bioactive mediators that influence body weight homeostasis, inflammation, and insulin sensitivity [43, 44]. These various protein signals are often referred to as adipocytokines and include adiponectin, leptin, IL-6, and TNF-α. Adiponectin levels are inversely related to indices of insulin resistance [45] and are low in patients with HIV-lipodystrophy [46, 47]. Low adiponectin levels are associated with a moderately increased CVD risk in diabetic men [48]. The differentiation of preadipocytes into mature adipocytes is a key process contributing to the normal function of adipose tissue. Subcutaneous adipocytes differ from visceral adipocytes in many respects. Compared with subcutaneous adipocytes, visceral adipocytes are hyperlipolytic and have a distinct secretion profile of adipocytokines [43, 44]. Lipodystrophy is characterized by impaired differentiation of preadipocytes to mature adipocytes [8, 9], resulting in reduced production of leptin and adiponectin [46]. Leptin deficiency and hypoadiponectinemia correlate with lipoatrophy and visceral fat accumulation in HIV-infected patients, whereas hypoadiponectinemia also appears to be associated with insulin resistance and dyslipidemia [47]. Clearly, these studies emphasize the importance of adipose tissue as an active endocrine organ involved in several metabolic and inflammatory processes that are relevant for the development of atherosclerosis. Both, excess of visceral fat and lack of subcutaneous fat, are related to impaired postprandial FFA

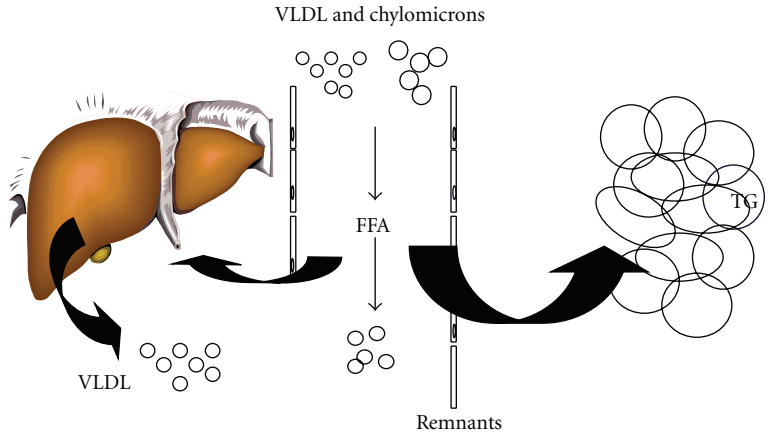

Figure 1: Postprandial lipid and FFA metabolism. In the postprandial phase, endogenous (VLDL) and exogenous (chylomicrons) TRL compete for clearance by LPL, which hydrolyzes TG into glycerol and FFA, leaving atherogenic remnant particles. The lipolytic rate, as well as the clearance of remnant particles by liver receptors, contributes to removal of TRL from the circulation. Adipose tissue plays a crucial role in regulating FFA concentrations by suppressing the release of FFA in the circulation and stimulating the uptake of FFA liberated from TRL by LPL. This pathway is known as the pathway of adipocyte FFA trapping.

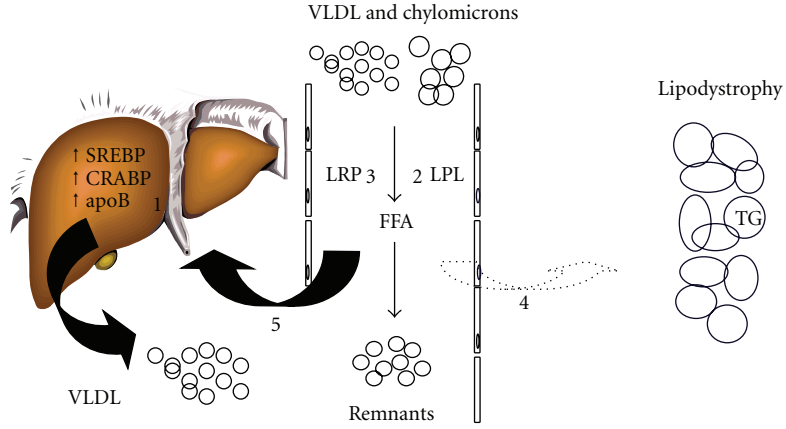

Figure 2: Pathogenesis of CART-related hyperlipidemia. Several factors contribute to hyperlipidemia in HIV-infected patients. PIs suppress the breakdown of the nuclear form of SREBP-1, as well as the proteasomal degradation of nascent apoB in the liver, leading to increased VLDL secretion (1). In the postprandial phase due to limited LPL availability, competition at the level of LPL will occur resulting in accumulation of TRL. This competition is most likely when fasting hypertriglyceridemia is present. In addition, impaired disposal of TRL is likely due to a defect in LPL activity (2) and delayed removal of remnant particles by liver receptors (3). Impaired FFA storage capacity (4) may lead to increased flux of circulating lipids (5) and upregulate hepatic VLDL production in patients with lipodystrophy.

handling, hypertriglyceridemia, and insulin resistance, which is partly mediated by adipocytokines.

6. Insulin Resistance

Insulin resistance is commonly seen in HIV-infected patients. Insulin resistance may result from direct effects of antiretroviral agents, effects of HIV infection, or indirect effects, such as changes in body fat distribution [49]. It has been shown that PIs induce insulin resistance in vitro by reducing insulin-mediated glucose uptake by glucose transporter 4 [50]. In HIV-negative adults, PIs reduce insulin sensitivity as early as 4 weeks after administration, without changing body fat distribution [17, 51, 52]. Direct effects of NRTIs and NNRTIs on insulin sensitivity have not been demonstrated, but these classes may contribute to insulin resistance indirectly through changes in body fat distribution. Insulin resistance has been related to excess of visceral fat, loss of subcutaneous fat, and increased waist-to-hip ratio [3–5]. Basal lipolytic rates are generally increased in patients with HIV-lipodystrophy, suggesting impaired action of hormone-sensitive lipase [53]. In addition, several studies have reported elevated FFA levels following glucose or insulin challenges, suggesting resistance to the action of insulin to suppression of lipolysis [54, 55]. The prevalence of insulin resistance among those treated with CART is up to 60 percent, depending on the criteria and techniques used [56, 57].

As a consequence of insulin resistance, abnormalities in glucose tolerance have been frequently observed in HIV-infected patients. The prevalence of diabetes mellitus (DM) was 7 percent in HIV-infected adults with lipodystrophy compared with 0.5 percent of healthy controls matched for age and BMI [58]. Impaired glucose tolerance was present in 35 percent of HIV-infected patients compared with 5 percent of matched controls [58]. The prevalence and incidence of DM have also been analyzed in the Multicenter AIDS Cohort Study [59]. In this study, 14 percent of HIV-infected men had DM compared with 5 percent of HIV-negative men (prevalence ratio of 4.4 after adjustment for age and BMI). In addition, DM was 3.1 times as likely to develop in HIV-infected men receiving CART as it was in control subjects over a three-year period of observation. Exposure to a PI-containing regimen, stavudine, or efavirenz was each independently associated with the development of DM.

7. Metabolic Syndrome

In the general population, several metabolic risk factors are strongly interrelated and are part of the metabolic syndrome (MS) as was elegantly described by Reaven in 1988 [60]. The MS encompasses disturbances in glucose, insulin, and lipid metabolism, associated with abdominal obesity. The National Cholesterol Education Program (NCEP) has endorsed the importance of the MS in cardiovascular risk assessment by introducing a case definition of the MS based on clinically easily obtainable anthropometric and laboratory parameters [61]. Using this definition, the MS is present when at least three out of five risk determinants (waist circumference $\geq$88 cm in women and $\geq$102 cm in men, TG $\geq$1.7 mmol/L, HDL-cholesterol $\leq$1.20 mmol/L in women or $\leq$1.0 mmol/L in men, glucose $\geq$6.1 mmol/L or blood pressure $\geq$130/85 mmHg) are present [61]. The MS affects 24 percent of the adult population in the U.S. [62] and 15 percent of nondiabetic adult Europeans [63] and is associated with an increased risk of CVD [64]. The MS is closely linked abdominal obesity [38], but absolute or partial lack of body fat may result in a similar metabolic risk profile [39–41].

Several studies focused on the prevalence and characteristics of the MS in HIV-infected patients and possible related factors. The prevalence of the MS ranges from 17 to 42 percent and is partly dependent on the definition used [65–71]. In a study of Jericó et al., the prevalence of the MS was 17 percent among HIV-infected patients and was independently associated with age, BMI, and past and present PI exposure [65]. In a report by Samaras et al., the prevalence of MS was 18 percent by NCEP criteria [66]. In this study, half of the patients had at least two features of MS but were not classified as having MS as their waist circumference was in the non-MS range. MS was more common in those currently receiving PIs and was associated with a substantially increased prevalence of DM in this specific cohort. In a study of Jacobsen et al., almost one quarter of the HIV-infected adults had the MS [67]. Most patients with the MS had low HDL-cholesterol and high TG plus $\geq$1 additional abnormality. The incidence of MS was higher with increasing viral load, higher BMI and higher trunk-to-limb fat ratio, and lopinavir/ritonavir or didanosine use. Mondy et al. reported that the overall prevalence of the MS was similar between HIV-infected patients (26 percent) and HIV-negative persons (27 percent), although the HIV-infected patients had a significantly smaller waist circumference, lower BMI, lower HDL-cholesterol, higher TG, and lower glucose levels, compared with the subjects from the NHANES cohort [68]. High prevalence of the MS was reported by three separate studies, ranging from 35 to 42 percent [69–71]. In the Data Collection on Adverse Events of Anti-HIV Drugs (DAD) study, for all definitions considered, there was an increasing prevalence of the MS over time, although the prevalence estimates themselves varied widely [71]. Using an NCEP definition that was modified to take account of the use of lipid-lowering and antihypertensive medication and measurement variability, an increase in prevalence from 19 percent in 2000/2001 to 42 percent in 2006/2007 was found.

The risk of developing the MS seems to be related to HIV-infection, specific antiretroviral agents and body fat distribution [65–71]. The MS in HIV-infected patients is diagnosed mostly through low HDL-cholesterol and high TG. Frequently, HIV-infected patients do not meet waist circumference criteria for the MS, despite high rates of body fat partitioning disturbances. The high lipodystrophy prevalence rates and skewing of BMI towards normal may partly explain the relatively low prevalence of the MS in HIV-infected patients compared with that of the general population, in which higher obesity rates are found. Thus, NCEP criteria may underestimate the prevalence of the MS in HIV-infected patients. It should also be noted that the presence of the MS in HIV-infected individuals does not appear to increase CVD risk over and above that conferred by the components of the MS separately [72]. Hence, it is unclear whether the MS has additive value in cardiovascular risk assessment of HIV-infected patients.

8. Surrogate Markers of Atherosclerosis

Endothelial dysfunction is an early marker of atherosclerosis and can be assessed clinically by ultrasound assessment of brachial artery flow-mediated vasodilation (FMD). FMD is correlated with the severity and extent of coronary sclerosis [73] and predicts future cardiovascular events [74]. Ultrasound measurement of carotid intima-media thickness (IMT) is a well-accepted, noninvasive method of assessing early changes in vascular structure and is widely used as a surrogate marker for atherosclerotic disease [75]. Assessment of both preclinical atherosclerotic markers may provide important information on the functional and structural stages of atherosclerosis.

In a cross-sectional study of HIV-infected adults, it was shown that those on a PI-containing regimen had markedly impaired FMD compared with those not taking PIs [76]. However, FMD was not compared with an HIV-negative reference group, and the relative contributions of CART, HIV infection, and metabolic risk factors were difficult to identify. Francisci et al. performed a retrospective

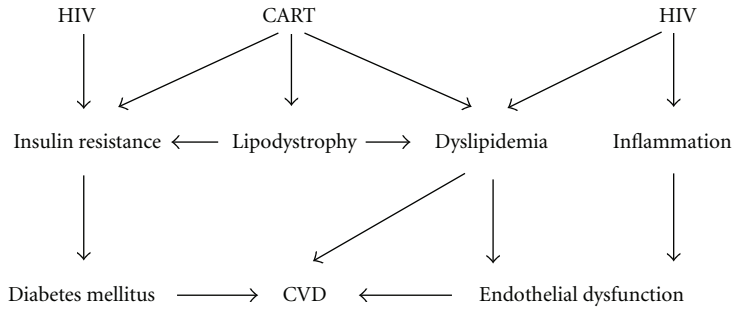

Figure 3: Pathogenesis of CVD in HIV-infected patients. HIV infection itself has been associated with subclinical atherosclerosis due to a low-grade inflammatory response leading to endothelial dysfunction. CART may promote atherosclerosis through its effects on body fat distribution, lipid metabolism and insulin sensitivity. Presumably, both HIV infection and CART promote atherosclerosis, either directly or indirectly via metabolic risk factors.

cohort study in HIV-infected patients before and after starting CART and matched healthy controls [77]. Soluble markers of endothelial function were significantly higher in HIV-infected patients before starting CART than in healthy controls. Short-term treatment with CART reduced some markers of endothelial dysfunction, with no differences between PIs and NNRTIs. In a prospective cross-sectional study by Ross et al., endothelial markers were also higher in CART-naive patients compared with healthy controls but were similar between HIV-patients on CART and healthy controls [78]. Strong correlations were found between inflammatory cytokines and endothelial markers. Hence, both studies highlight a potential association between inflammation and endothelial activation [77, 78]. The endothelium could be activated either directly by HIV or by a leukocyte-mediated inflammatory cascade triggered by HIV infection [79–81]. In recent years, a prominent role for inflammation in the pathogenesis of atherosclerosis has emerged and circulating inflammatory molecules have been identified as markers of atherosclerotic risk [82]. Antiretroviral agents may also directly induce endothelial dysfunction. For example, when healthy volunteers were given the PI indinavir for 4 weeks, significant endothelial dysfunction was observed, independent of the lipid profile [83, 84]. Unlike the dramatic impairment seen with indinavir, the newer PIs atazanavir and lopinavir/ritonavir did not induce endothelial dysfunction in healthy subjects [85]. Finally, endothelial dysfunction has also been related to metabolic risk factors. In one study, the presence of the MS in HIV-infected patients was associated with markedly impaired FMD [69]. FMD was related to several metabolic parameters, such as dyslipidemia and insulin resistance [69].

Structural vascular abnormalities are also present in HIV-infected patients. Carotid IMT is higher in HIV-infected patients than in age-matched controls [86, 87], and progresses much more rapidly than previously reported rates in non-HIV cohorts [88]. In HIV-infected patients, IMT is related to several traditional risk factors [69, 86–88] but when a control group was added to the analysis, HIV infection was also an independent predictor of IMT [88]. Furthermore, progression of IMT has been related to low nadir CD4 cell counts [88]. Even after adjustment for traditional risk factors, HIV-infected patients have greater carotid IMT than controls [89]. In one study, the association between HIV infection and carotid IMT was similar to that of traditional risk factors, such as smoking [89]. Inflammatory and endothelial activation markers have been associated with increased carotid IMT, supporting a potential role of inflammation in CVD in HIV-infected patients [90]. Increased carotid IMT has also been related to CART. In a cross-sectional study of Jericó et al., 42 percent of the HIV-infected patients had subclinical carotid atherosclerosis, defined by carotid IMT >0.8 mm or the presence of plaque [91]. Exposure to CART was independently associated with subclinical carotid atherosclerosis in this study. Finally, HIV-infected individuals with the MS may be at increased risk for atherosclerosis based on higher carotid IMT [69, 92]. HIV-infected patients with the MS were more likely to have a carotid IMT >0.8 mm than were those without MS. Any positive coronary artery calcium score was more likely to occur for participants with MS [92].

Taken together, most studies support the concept that HIV-infected patients are at risk for accelerated atherosclerosis. As illustrated in this section, the underlying mechanism is probably multifactorial, which is schematically depicted in Figure 3. The HIV infection itself may directly induce insulin resistance and dyslipidemia, including hypertriglyceridemia and low HDL-cholesterol. Furthermore, chronic HIV infection is associated with a proinflammatory state leading to endothelial dysfunction. CART may also promote atherosclerosis through mechanisms involving endothelial cells, either directly or indirectly via metabolic risk factors.

9. Cardiovascular Disease

Use of CART in HIV-infected patients has been associated with a large benefit in terms of mortality [1, 2]. In a large retrospective study, this benefit was not diminished by any increase in the rate of CVD [1]. However, this study was conducted among 36,766 patients who received care for HIV infection between 1993 and 2001, and longer-term observations and analyses are required. Since then, several studies on CVD endpoints have been published, of which most demonstrate increased CVD risk in HIV-infected patients. In the DAD study, CART was independently

associated with a 26 percent relative increase in the rate of myocardial infarction (MI) per year of exposure during the first four to six years of use [6]. However, the absolute risk of MI was relatively low. Hypercholesterolemia, older age, smoking, DM, male sex, and a prior history of CVD were also associated with an increased risk of MI [6]. A central question is whether this observed risk is attributable to all classes of antiretroviral drugs or only to specific drugs. Subsequent analyses of the DAD study have demonstrated that particularly those exposed to PIs and those recently exposed to the NRTIs abacavir and didanosine had increased risk of MI [93, 94]. In contrast, no association was found between the risk of MI and exposure to NNRTIs or any of the other NRTIs [93–96]. The effect of PIs may be in part a consequence of the effects of these agents on lipid levels [93]. In contrast, associations between MI risk and abacavir and didanosine exposure were largely confined to those patients with recent exposure to the drugs and did not appear to be driven by dyslipidemia [94, 96]. Abacavir may cause vascular inflammation [96].

Triant et al. conducted a health care system-based cohort study using a large data registry with 3,851 HIV and 1,044,589 non-HIV patients [7]. MI rates were determined among patients receiving longitudinal care between 1996 and 2004. MI rates and cardiovascular risk factors were increased in HIV compared with non-HIV patients. The relative risk of acute MI was 1.75 in HIV-infected patients after adjustment for age, sex, race, hypertension, diabetes, and dyslipidemia. The increased MI event rate was seen over multiple age ranges and, thus, likely to be clinically significant. It should be noted that the rate of MI was higher among HIV patients in this study than in the DAD study, but this study included older patients and was from a U.S. population, with potentially different MI rates and cardiovascular risk factors than the European-based population of the DAD study.

Inflammation appears to be an important pathogenic event in the progression of atherosclerosis [82]. Premature atherosclerosis has been reported in young adults with HIV infection in the pre-CART era [97]. Also, interruption of CART seems to be associated with an increased short-term risk of CVD [98]. Infection-induced chronic inflammation may thus contribute to the increased incidence of CVD in HIV-infected patients. In line, low CD4 cell counts have been associated with incident CVD in the HIV Outpatient Study [99]. CD4 cell count $\leq$500 cells/mm^3 was an independent risk factor for incident CVD, comparable in attributable risk to several traditional CVD risk factors [99]. Thus, traditional risk factors, HIV infection, and antiretroviral agents have all been associated with CVD endpoints in HIV-infected patients.

10. Treatment of Risk Factors

Dyslipidemia and insulin resistance are important modifiable risk factors in HIV-infected patients. Preliminary data indicate increased cardiovascular morbidity among HIV-infected patients, suggesting that measures to reduce cardiovascular risk should be provided. It has been recommended that HIV-infected adults undergo evaluation and treatment on the basis of NCEP guidelines for dyslipidemia, with particular attention to potential drug interactions with antiretroviral agents and maintenance of virological control of HIV infection [61, 100]. In general, treatment guidelines outlined by the American Diabetes Association (ADA) and European Association for the Study of Diabetes (EASD) should be followed in HIV-infected patients with DM.

10.1. Lifestyle Modification. Cigarette smoking is the most important modifiable risk factor among HIV-infected patients. In the DAD study, more than 50 percent of the patients were current or former smokers, and smoking conferred a more than 2-fold risk of MI [6, 12]. Cessation of smoking is likely to reduce CVD in this population. Management of dyslipidemia must include nondrug interventions, such as a prudent diet, reduced total caloric intake, attaining ideal bodyweight, and increased physical activity. Routine aerobic activity and muscle conditioning improved trunk adiposity and lipid parameters in HIV-infected patients [101–103]. A recent randomized study showed that dietary intervention in CART-naive HIV-infected patients prevented development of dyslipidemia after 6 and 12 months [104]. Structured exercise plus diet decreased total cholesterol and TG by 11% and 21%, respectively, in HIV-infected patients [105]. HIV-infected patients with hypertriglyceridemia may also benefit from omega fatty acids [106, 107].

10.2. Switching Cart. Another strategy to improve dyslipidemia is switching antiretroviral agents. Switching antiretroviral agents has the potential advantage of avoiding pharmacologic intervention for elevations in lipid levels. Switching from the PI nelfinavir to the PI atazanavir reduced total cholesterol and TG with no apparent antiviral compromise [108]. Other studies have shown that switching a PI for either an NNRTI or NRTI, such as nevirapine, efavirenz, or abacavir, in patients with long-lasting viral suppression has antiviral efficacy similar to earlier PI-based combinations and may partly reverse atherogenic lipoprotein changes [109–113].

10.3. Lipid-Lowering Agents. Because of the potential for significant drug interactions with commonly used antiretroviral drugs, the choices of lipid-lowering agents should be limited to those agents with a low likelihood of interactions. HMG-CoA reductase inhibitors or statins are used as first-line therapy for hypercholesterolemia and reduce the risk of CVD in the general population. Several statins have been studied in HIV-infected patients. For patients receiving CART or other medications that inhibit CYP3A4, lovastatin and simvastatin should be avoided, and atorvastatin and rosuvastatin should be used with caution. In CART-treated HIV-infected patients, treatment with pravastatin was associated with improvement of the lipid profile and endothelial function [114, 115]. Others have suggested that atorvastatin and rosuvastatin are preferable to pravastatin for treatment of HIV-associated dyslipidemia, due to greater reductions in LDL-cholesterol and non-HDL-cholesterol, with similar low toxicity rates [116]. In this report, the likelihood of reaching NCEP goals for LDL-cholesterol levels was higher

with the use of rosuvastatin (OR 2.1) and atorvastatin (OR 2.1) compared with that of pravastatin. A recent analysis of 829 patients has shown that dyslipidemia is more difficult to treat in HIV-infected patients than in the general population, as illustrated by smaller reductions in LDL-cholesterol and TG with lipid-lowering agents [117].

Fibrates, synthetic agonists for PPAR-α, have a well-established tolerability and efficacy profile for patients with hypertriglyceridemia and mixed hyperlipidemia. Gemfibrozil, fenofibrate, and bezafibrate have been associated with improvements of the lipid profile in HIV-infected patients [118–120]. There are no significant drug-drug interactions among PIs and fibrates. So far, the results of clinical trials on CVD endpoints with fibrates have been disappointing. However, the absolute benefits of fenofibrate are likely to be greater when MS features, including hypertriglyceridemia, are present [121]. Thus, fibrates would seem to be the preferred treatment for HIV-infected patients with dyslipidemia characterized mainly by hypertriglyceridemia. At the present time, there is no compelling reason to prefer fenofibrate to gemfibrozil in HIV-infected patients. Modest LDL-cholesterol lowering with ezetimibe has also been observed in HIV-infected patients, although its effect on CVD endpoints is unclear [122, 123].

10.4. Insulin-Sensitizing Agents. Because of the severity of insulin resistance in many HIV-infected patients with DM, it is reasonable to favor insulin sensitizers over insulin secretagogues. Insulin-sensitizing agents have also been studied in nondiabetic HIV-infected patients. In patients with lipoatrophy, metformin should be used with caution because further reductions in subcutaneous fat may be seen. On the other hand, studies with metformin have demonstrated significant reduction of visceral fat and improvement of insulin sensitivity, lipid levels, and endothelial function [124–127]. Thiazolidinediones, synthetic agonists for PPAR-γ, can be considered the preferred approach in those with lipoatrophy, given the possibility of increasing subcutaneous fat, albeit modest [127–130]. Of the thiazolidinediones, rosiglitazone improves insulin sensitivity, but most studies found detrimental effects on lipid levels [127–130]. In one study, rosiglitazone improved postprandial adipocyte FFA trapping but caused a marked increase in postprandial remnant lipoprotein levels, which may adversely affect cardiovascular risk [131]. The other registered thiazolidinedione, pioglitazone, also improves insulin sensitivity and is associated small benefits on fasting lipid profile in HIV-infected patients [132, 133]. However, pioglitazone is partly metabolized by CYP3A4, increasing the risk of clinically relevant drug interactions with PIs.

11. Conclusions

In HIV-infected patients, the use of CART is associated with changes in body composition, dyslipidemia, and insulin resistance. Disturbed adipose tissue distribution and altered secretion of adipocytokines may play a key role in the development of hypertriglyceridemia and insulin resistance. Presumably, both HIV infection and CART may contribute to increased CVD risk in HIV-infected patients. The absolute CVD risk, however, is still relatively small and side effects of CART should be balanced against the large benefit in terms of AIDS-related mortality. Nonetheless, as HIV-infected patients live longer on CART, CVD could become increasingly prevalent in the future. Guidelines for the evaluation and treatment of dyslipidemia have been provided. Current treatment options include lifestyle modification, switching antiretroviral agents, and use of lipid-lowering and insulin-sensitizing agents. Future research will give more insight into the pathophysiology of CVD in HIV-infected patients and the role of CART and adipose tissue.

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Research Article

Phospholipase A2 Mediates Apolipoprotein-Independent Uptake of Chylomicron Remnant-Like Particles by Human Macrophages

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Apolipoprotein E-receptor-mediated pathways are the main routes by which macrophages take up chylomicron remnants, but uptake may also be mediated by receptor-independent routes. To investigate these mechanisms, triacylglycerol (TG) accumulation induced by apolipoprotein-free chylomicron remnant-like particles (CRLPw/o) in human monocyte-derived macrophages was evaluated. Macrophage TG content increased about 5-fold after incubation with CRLPw/o, and this effect was not reduced by the inhibition of phagocytosis, macropinocytosis, apolipoprotein E function, or proteoglycan bridging. The role of lipases, including lipoprotein lipase, cholesteryl ester hydrolase, and secretory (sPLA2) and cytosolic phospholipase A2, was studied using [^{3}H]TG-labelled CRLPw/o. Total cell radioactivity after incubation with [^{3}H]TG CRLPw/o was reduced by 15–30% by inhibitors of lipoprotein lipase and cholesteryl ester hydrolase and by about 45% by inhibitors of sPLA2 and cytosolic PLA_2 . These results suggest that macrophage lipolytic enzymes mediate the internalization of postprandial TG-rich lipoproteins and that $sPLA_2$ and cytosolic PLA2, play a more important role than extracellular lipoprotein lipase-mediated TG hydrolysis.

1. Introduction

Lipids from the diet are absorbed from the intestine in chylomicrons, large triacylglycerol (TG)-rich lipoproteins, which are secreted into lymph and pass into the blood via the thoracic duct. The chylomicrons then undergo rapid lipolysis by lipoprotein lipase (LPL) in extrahepatic capillary beds, a process which removes some of the TG and leaves smaller chylomicron remnants which deliver the remaining dietary lipids to the liver [1]. It was believed for many years that chylomicron remnant size prevented their entrance into the artery wall and their subsequent interaction with macrophages. However, it is now clear that chylomicron remnants can penetrate and be retained within the subendothelial space as efficiently as low density lipoprotein (LDL) [2, 3]. In addition, apolipoprotein-B48-containing lipoproteins have been isolated from atherosclerotic plaques [4].

Chylomicron remnants have been shown to be taken up by several types of macrophages and to cause extensive TG and cholesterol accumulation leading to foam cell formation [5–8], further supporting the atherogenic role of this lipoprotein. The pathways mediating macrophage uptake of chylomicron remnants are apolipoproteinE (apoE)-dependent receptor-mediated processes involving the LDL receptor and the LDL receptor-related protein (LRP) [9–11]. However, several studies have found evidence that chylomicron remnant uptake pathways in these cells may be independent of the LDL receptor [6, 12] and apoE production [6]. Furthermore, Fujioka et al. [6] have reported that apolipoprotein-free remnant particles are taken up and promote lipid deposition in macrophages from apoE-deficient mice [6]. Thus, it seems likely that there are non-apoE-mediated receptor pathways which mediate the uptake of apolipoprotein-free chylomicron remnants by human

macrophages. The aim of this study was to investigate these pathways and to evaluate whether secretory lipases are involved in their function.

2. Materials and Methods

2.1. Materials. Glycerol-tri[9,10(n)-^{3}H]oleate (28 Ci/mmol), [1(3)-^{3}H]glycerol (60 mCi/mmol), and [4-^{14}C]cholesteryl-oleate (60 mCi/mmol) were obtained from NEN Life Science Products Inc. Boston, Mass, USA. Iscove's Modified Dulbecco's Medium (IMDM), fetal bovine serum (FBS), Ficoll-Paque, penicillin, and streptomycin were obtained from Hyclone Europe Ltd., CD14 MicroBeads and LS Separation Columns were purchased from Miltenyi Biotech. Goat antibody to human apoE and goat immunoglobulin G (IgG) were obtained from Biodesign (Bologna, Italy). Cytochalasin D, orlistat, brefeldin, manoalide, fatty acid-free bovine serum albumin (BSA), phorbol 12-myristate 13-acetate (PMA), heparinase I, heparinase III, MJ33, sodium chlorate, and various classes of lipids and solvents were purchased from Sigma Chemical Company (St. Louis, Mo, USA). methyl arachidonyl fluorophosphonate (MAFP) and haloenol lactone suicide substrate (HELSS) were purchased from Biomol International (Vinci-Biochem, Vinci, Italy). For lipid analysis of lipoprotein particles, enzymatic kits for the determinations of total (TCH) and free cholesterol (FCH) were obtained from WAKO (Test Medical, Zola, Italy) and that for TG from BPC (Rome, Italy).

2.2. Macrophages. Monocytes were isolated from human buffy coats as previously described [13]. Buffy coats from the blood of healthy donors were diluted 1 : 3 with phosphate-buffered saline (PBS) and layered on Ficoll-Paque. After centrifugation, white blood cells were collected and washed with PBS. CD14 MicroBeads were used for the positive selection of human monocytes from white blood cells. According to the manufacturer's instructions, 300–400 $\times 10^6$ total cells, magnetically labelled with CD14 MicroBeads, were applied to LS Separation columns, and the total effluent was discarded. Monocytes (CD14-positive fraction), flushed out of the column, were washed, and 1.5×10^6 cells transferred to 22-mm dishes at a concentration of 8×10^5 cells/mL and cultured in IMDM containing 15% FBS. The purity of isolated monocytes, monitored by specific flow cytometric analysis for CD14, ranged 95–97%. The differentiation process from monocytes to macrophages was monitored by the increased expression of CD71 antigen. The experiments were performed with human monocyte-derived macrophages (HMDM) 10 days after plating. For experiments involving the measurement of lipid accumulation, CRLP were incubated with HMDM for 24 h, so that there was sufficient uptake to allow accurate determination of TG and cholesterol in the cells. Shorter incubation times (5-6 h) were used for experiments involving radioactivity, as these techniques are more sensitive.

Cultures of the J774.2 murine macrophage-like cell line (J774) were obtained from the American Type Culture Collection (Rockville, Md, USA). Cells were maintained in DMEM supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL), glutamine (2 mM), and 10% FBS at 37°C in a humidified atmosphere of 95% air/5% CO_2. For experiments, cells (at passage 6–10) were seeded into 22-mm dishes at a concentration of 10^5 cells/mL and used on the 3rd day of culture.

2.3. Preparation of CRLP. Chylomicron remnant-like particles (CRLP) containing TG as the major lipid class were prepared by sonication of a lipid mixture followed by ultracentrifugation [14]. A lipid mixture containing 70% triolein (18 : 1), 2% cholesterol, 3% cholesteryl ester, and 25% phospholipids (70.5% phosphatidylcholine, 11% phosphatidylethanolamine, 6.9% lysophosphatidylcholine, 6.5% sphingomyelin, 2.6% phosphatidylinositol, and 2.6% phosphatidylserine) was sonicated in 0.9% NaCl in Tricine buffer (20 mM, pH 7.4) for 20 min at 37°C with a power giving an amplitude range, quoted as total peak to peak moments of 22–24 μm (Branson 250/450 sonifier). For the preparation of CRLPw/o with labelled TG, before sonication, 100 μCi of glycerol-tri[^{3}H] oleate ([^{3}H]TG) was added to the lipid mixture. After sonification, the density of the emulsion was increased to 1.21 g/mL with KBr, layered under a step-wise density gradient and centrifuged at 17,000 g for 20 min at 20°C. The upper layer was discarded and replaced with an equal volume of KBr (d 1.006 g/mL), and the centrifugation was repeated at 70,000 g for 1 h at 20°C. The particles were harvested from the top layer, dialyzed against medium without FBS, but containing penicillin/streptomycin. These CRLP (CRLPw/o), which do not contain apolipoproteins, were used for experiments within 2 days of their preparation. For the preparation of lipid particles containing apoE (CRLP+), CRLPw/o were incubated with human plasma as previously described [13]. Previous analysis by SDS-PAGE has shown that CRLP prepared in this way contain apoE and no other apolipoproteins [11]. The band corresponding to apoE was not detected in lipid particles prior to incubation with plasma, nor in the top fraction from plasma centrifuged in the absence of lipid particles, indicating that the CRLP acquired apoE during the incubation.

2.4. Negative Staining Electron Microscopy of CRLP. Samples were diluted with distilled water to achieve a satisfactory concentration of lipid particles for negative staining. A 200-mesh nickel grid with a type-B carbon support film (Ted Pella, Redding, Calif, USA) was incubated with a drop of diluted sample. After 10 minutes, the grid was drained with filter paper and stained with 2% phosphotungstic acid (pH = 4) for 2 minutes. The grid was drained and photographed with a Jeol 1200 transmission electron microscope.

2.5. Cellular Assay of Cholesterol and TG. After incubations, cells were washed 3 times with PBS and harvested from wells by scraping into 500 μL of distilled water. An aliquot of cellular suspension was utilized to determine protein content by Lowry's method [15], using BSA as a standard. After the extraction of cellular lipids [16], the TCH and TG content of cells was determined by fluorimetric methods according to

Gamble et al. [17] and Mendez et al. [18], respectively. Each determination was performed in duplicate.

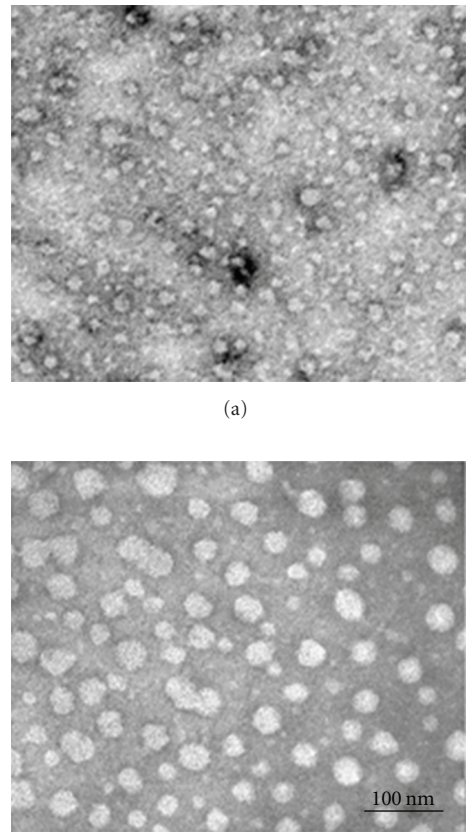
2.6. Assay of TG Synthesis. Synthesis of TG and PL was evaluated by determining the incorporation of [^{3}H]glycerol into TG and PL as previously described [13]. HMDM were incubated for 5 h at 37°C in serum-free IMDM containing 80 μg cholesterol/mL CRLPw/o in the presence of [^{3}H]glycerol (4 μCi/mL, 20 μM). After the incubations, HMDM were washed 3 times with PBS, and cell lipids were extracted with hexane/isopropanol; 3 : 2, v/v. [^{14}C]CE was added as an internal standard and the lipids classes were separated by thin layer chromatography on silica gel (Merck, Germany) developed in hexane/ether/acetic acid (70 : 30 : 1, v/v/v). Radioactivity associated with the bands corresponding to [^{3}H]TG and [^{3}H]PL were scraped from the plates and assayed for radioactivity in an LS5000 Beckman liquid scintillation counter.

2.7. Statistical Methods. Repeated Measure Analysis of variance (ANOVA) and multiple comparisons using the Tukey-Kramer Multiple Comparison Test or Student's paired t-test were used to evaluate significant differences in the means between groups. $P < 0.05$ was considered significant.

3. Results

3.1. Characterization of CRLP. Biochemical characterization of CRLPw/o and CRLP+ (Table 1) showed that CRLPw/o and CRLP+ differ in their TG/TCH (9.68 ± 2.88 versus 4.95 ± 2.30, respectively; $P < 0.005$) and FCH/TCH (0.52 ± 0.30 and 0.21 ± 0.11, resp.; $P < 0.05$) molar ratios. As the TG concentration was not different between CRLPw/o (8.41 ± 2.9 mM) and CRLP+ (7.42 ± 3.79 mM), much of the difference between the lipid ratios are attributable to a decreased TCH content in CRLP-w/o in comparison with CRLP+ (0.87 ± 0.57 mM versus 1.79 ± 0.88 mM, $P < 0.005$). These changes in biochemical composition were accompanied by differences in the size of the particles. The average particle diameter determined from electron microscopy of CRLPw/o (Figure 1(b)), and CRLP+ (Figure 1(a)) was 34 ± 5 nm and 24 ± 7 nm, respectively. Preparations of [^{3}H]-TG CRLPw/o produced particles with a specific activity of 2780 and 3810 dpm/nmol of fatty acid in the 2 different preparations used for the 3 experiments. Radioactivity distribution in the lipids carried by the particles was similar in the 2 preparations. More than 90% of the radioactivity carried by [^{3}H]-TG CRLP was associated with TG, while the radioactivity associated with PL, free fatty acids, cholesteryl ester, and the sum of radioactivity recovered from the TLC plate but not associated with these bands was 2.8, 2.1, 0.4, and 4.7 %, respectively.

3.2. Role of Plasma-Derived Factors in Macrophage Lipid Accumulation Induced by CRLP. To investigate the role of plasma-derived factors in the induction of human macrophage lipid accumulation by chylomicron remnants, we measured macrophage TG and TCH of macrophages incubated with either CRLP+ or CRLPw/o in comparison to macrophages incubated without the lipid particles. The small consistent increase (12%) of cell cholesterol (Figure 2(a)) induced by CRLP+ (89.4 ± 4.5 nmol/mg protein, $n = 6$; $P < 0.05$ versus control w/o) in comparison with the control (79.1 ± 4.9 nmol/mg protein), was not observed with CRLPw/o (83.9 ± 7.4 nmol/mg protein). The presence of CRLP+ induced almost a 10-fold increase in TG macrophage content (47.8 ± 15.8 and 464.3 ± 190.2 nmol/mg protein in the absence or presence of CRLP+, resp.), and this was reduced to about 5 fold (226.3 ± 93.1 nmol/mg protein), (Figure 2(b)), when CRLPw/o particles lacking apolipoproteins were used. Thus, the increase in macrophage TG content after incubation with CRLPw/o remained substantially higher than the control ($P < 0.05$), indicating that CRLPw/o can induce macrophage lipid accumulation independent of plasma-derived apolipoproteins.



(a)

(b)

Figure 1: Negative staining electron microscopy (EM). Negatively stained CRLPw/o (b) and CRLP+ (a). The particle diameter expressed as mean ± SD was 34 ± 5 nm for CRLPw/o (190 particles) and 24 ± 7 nm for CRLP+ (160 particles).

3.3. Role of Phagocytosis and Macropinocytosis in Macrophage Internalization of CRLPw/o. Macrophages are professional phagocytes which carry out two related uptake processes, phagocytosis, and macropinocytosis [19], both of which may be involved in atherosclerosis development [20, 21].

Table 1: Biochemical characterization of chylomicron remnant-like lipid particles (CRLP) incubated without (CRLPw/o) or with plasma (CRLP+). Total cholesterol (TCH), triacylglycerol (TG), and free cholesterol (FCH) are expressed as means ± SD (n).

	TCH (mM)	TG (mM)	FCH (mM)	TG/TCH (molar ratio)	FCH/TCH (molar ratio)
CRLPw/o	0.87 ± 0.57 (20)**	8.41 ± 2.90 (20)	0.45 ±0.21 (7)	9.68 ± 2.88 (20)**	0.52 ± 0.30 (7)*
CRLP+	1.79 ± 0.88 (5)	7.42 ± 3.79 (5)	0.38 ± 0.20 (5)	4.95 ± 2.30 (5)	0.21 ± 0.11 (5)

* $P < 0.05$ versus CRLP+
** $P < 0.005$ versus CRLP+.

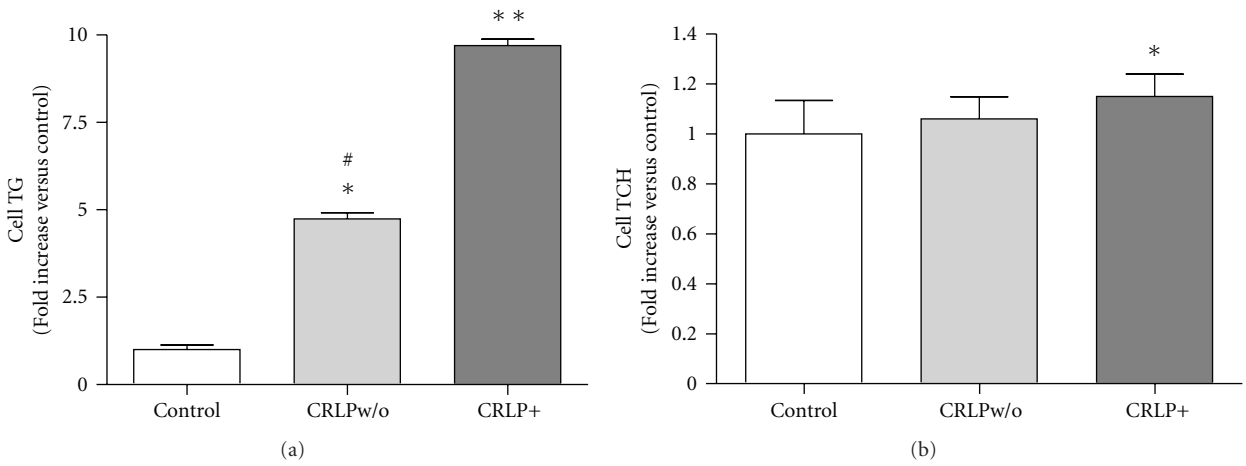


Figure 2: Effect of plasma treatment of CRLP on macrophage lipid accumulation induced by CRLP. HMDM were incubated for 24 h in serum-free medium without the addition of CRLP (control) or with 80 μg cholesterol/mL of either CRLP+ (contains apoE) or CRLPw/o (lacks apoE). (a) Cellular TG; (b) total cholesterol (TCH) content was determined by fluorimetric assay (mean ± SD, $n = 6$). Results are expressed as increase of lipid content with respect to the control. * $P < 0.05$, ** $P < 0.001$ versus control; # $P < 0.05$ versus CRLP+.

Phagocytosis and macropinocytosis can be blocked by cytochalasin D, and macropinocytosis can be induced in some macrophage phenotypes by treatment with phorbol esters such as PMA [20]. Thus, we investigated the function of these pathways on CRLPw/o uptake by incubating HMDM with CRLPw/o 20 μg (results not shown) or 80 μg cholesterol/mL) in the presence of cytochalasin D (2 μg/mL), or PMA (1 μg/mL), or the vehicle alone (control). The results (Figure 3) show that the changes in cellular TG and TCH content caused by CRLPw/o were not modified by cytochalasin D or PMA, suggesting that, over the concentration range of CRLP tested, phagocytosis and macropinocytosis are not directly involved in CRLPw/o-induced macrophage lipid accumulation.

3.4. Effect of Inhibition of Macrophage Secretion on TG Accumulation. Macrophages display a wide range of functions and secrete many factors potentially affecting lipoprotein metabolism (i.e., apoE and lipases) that could mediate uptake of CRLPw/o. To evaluate if factors secreted by macrophages contribute to the internalization of CRLPw/o, we added CRLPw/o (80 μg cholesterol/mL) to macrophages that had been preincubated for 3 h with 0 (control), 5 and 15 μg/mL brefeldin, an early stage inhibitor of the secretory pathway [22]. Incubation was then continued for 24 h in the presence of brefeldin. Macrophage TG content (542 ± 142 nmol TG/mg protein in the absence of brefeldin) was significantly ($P < 0.05$, $n = 6$) reduced by about 39% and 31%, respectively, by 5 and 15 μg/mL of brefeldin (434 ± 139 and 489 ± 126 nmol TG/mg protein with 5 and 15 μg/mL of brefeldin, resp.) compared with the control, suggesting that secretory factors produced by macrophages contribute to TG accumulation induced by CRLPw/o in macrophages.

3.5. Macrophage apoE Secretion and Internalization of CRLPw/o. The absence of plasma-derived apoE in CRLPw/o reduced but did not prevent macrophage lipid accumulation induced by the lipid particles (Figure 2). However, apoE is secreted in large amounts by human macrophages [23]; thus, CRLPw/o uptake could be mediated via the acquisition of the apolipoprotein during the incubation. To test this hypothesis, we measured the effects of an apoE antibody (apoE-Ab) on macrophage TG accumulation induced by CRLPw/o. For this purpose, HMDM were incubated for 24 h with CRLPw/o (80 μg cholesterol/mL) in the presence of 100 μg/mL of apoE-Ab or control IgG isotype. The TG content of macrophages at the end of incubations with apoE-Ab (708.9 ± 152.6 nmol/mg protein) was not different from incubations with IgG (842 ± 310 nmol/mg protein) or in the absence of antibody (control: 703.5 ± 213.5 nmol/mg protein) ($n = 3$), indicating that apoE secreted by macrophages does not account for the uptake of the apoE-free CRLPw/o.

To further ascertain whether the secretion of apoE has a role in CRLPw/o lipid uptake, we carried out experiments

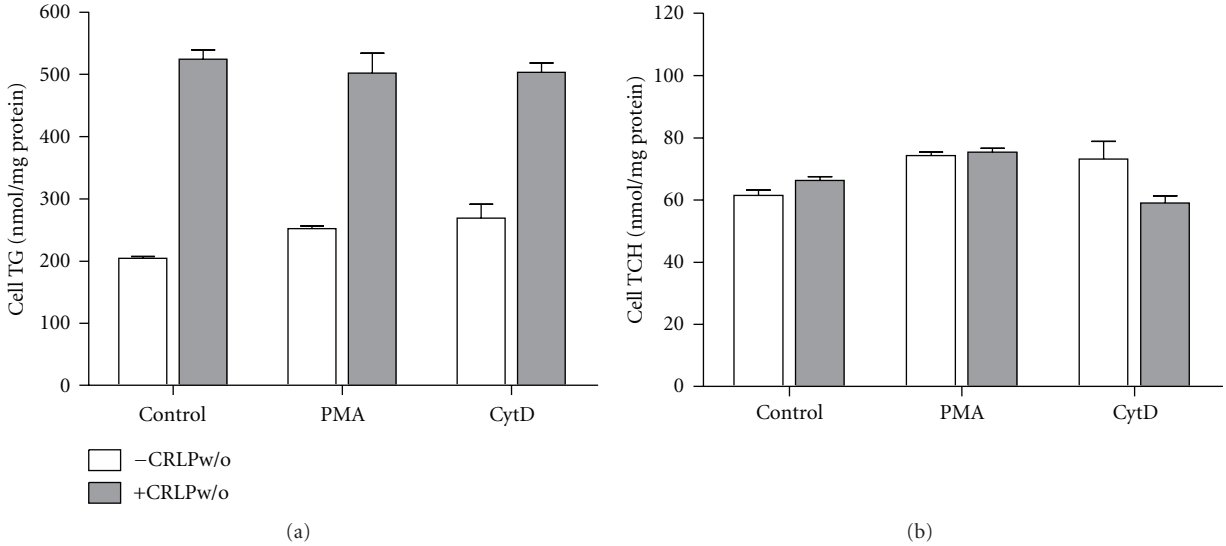


Figure 3: Effect of cytochalasin D and PMA on macrophage lipid accumulation. HMDM were incubated 24 h with 80 μg cholesterol/mL CRLPw/o or without CRLPw/o in the presence of 2 μg/mL cytochalasin D (CytD), or 1 μg/mL PMA both dissolved in DMSO or DMSO alone (control). Macrophage triacylglycerol (TG) and total cholesterol (TCH) content (nmol lipid/mg protein) were determined by fluorimetric assay (mean ± SD, $n = 4$).

with J774 cells, a murine macrophage cell line which secretes extremely low levels of apoE [24]. In control J774 macrophages, similar to the results with HMDM, CRLPw/o (80 μg cholesterol/mL) induced a significant increase in TG content (without CRLPw/o, 132.9 ± 21.9; with CRLPw/o 230.8 ± 10.9 nmol/mg protein; $P < 0.05$, $n = 3$) with no significant change in cholesterol content. Thus, the relative lack of apoE secretion did not prevent J774 macrophages from accumulating TG in the presence of CRLPw/o, confirming that apoE is not necessary for the uptake of these particles by the cells.

3.6. Role of Proteoglycan Bridging in Lipid Accumulation Induced by CRLPw/o. The interaction of lipoproteins with arterial proteoglycans facilitates the retention and the metabolism of TG-rich lipoproteins. In particular, proteins secreted by macrophages, such as apoE, lipoprotein lipase (LPL), and the secretory phospholipase A2 ($sPLA_2$), independently of their function, can act as structural cofactors facilitating cellular uptake of whole lipoprotein particles. These molecules can bridge between lipoproteins and heparan sulfate proteoglycans, concentrating lipoproteins in the vicinity of receptors or promoting entry of lipoproteins during the process of cell surface proteoglycan internalization [25, 26]. If this process was involved in CRLPw/o internalization by macrophages, therefore, the inhibition of the interaction with proteoglycans would be expected to impair their uptake. To test this hypothesis, HMDM, untreated (control) or pretreated for 1 h with heparinase I (33 U/mL) [27] or heparinase III (33 U/mL), both with 50 mM Na chlorate to inhibit proteoglycan synthesis [28], were further incubated for 24 h with CRLPw/o (80 μg cholesterol/mL). As reported in Figure 4, a rise (rather than a decrease) in TG content was induced by both heparinase III and heparinase I (586 ± 71 and 539 ± 58 nmol/mg protein, resp.) in comparison with the control group (445 ± 64 nmol/mg protein). Thus, the disruption of cell surface proteoglycans with heparinase did not decrease CRLP TG uptake by macrophages, indicating that the function of LPL, and other secreted proteins in bridging with proteoglycan is not involved in CRLPw/o internalization by macrophages.

3.7. Role of Macrophage Lipase Activities on the Internalization of TG Carried by CRLPw/o. The experiments above show that CRLPw/o induces the accumulation of TG but do not provide information about the mechanism involved. However, the data obtained with brefeldin suggests that macrophage-secreted factors may function in CRLPw/o TG uptake. Among the proteins secreted by macrophages, there are a number of lipases, including LPL, $sPLA_2$, and cholesteryl ester hydrolase [29–32]. Secreted LPL has been shown to have an important function in the uptake of fatty acids derived from the extracellular lipolysis of TG carried by TG-rich lipoproteins [29]. In view of this, we examined whether the lipases produced by macrophages contribute to TG uptake from CRLPw/o. Macrophage $sPLA_2$ cleave phospholipid (PL) fatty acids in the sn-2 position [31], and, while fatty acids released by cholesteryl ester hydrolase secreted by macrophages derive mainly from cholesteryl ester, TG and PL may also function as substrates [31]. The fatty acids liberated by the catalytic action of extracellular lipase, however, could supply substrate for cellular TG synthesis, even without internalization of whole CRLPw/o. Alternatively, fatty acids released by these lipases could occur after the internalization of CRLPw/o. In order to evaluate whether macrophage lipolytic activities contribute to the internalization of intact TG carried by CRLPw/o, we used an experimental approach involving radiolabelled

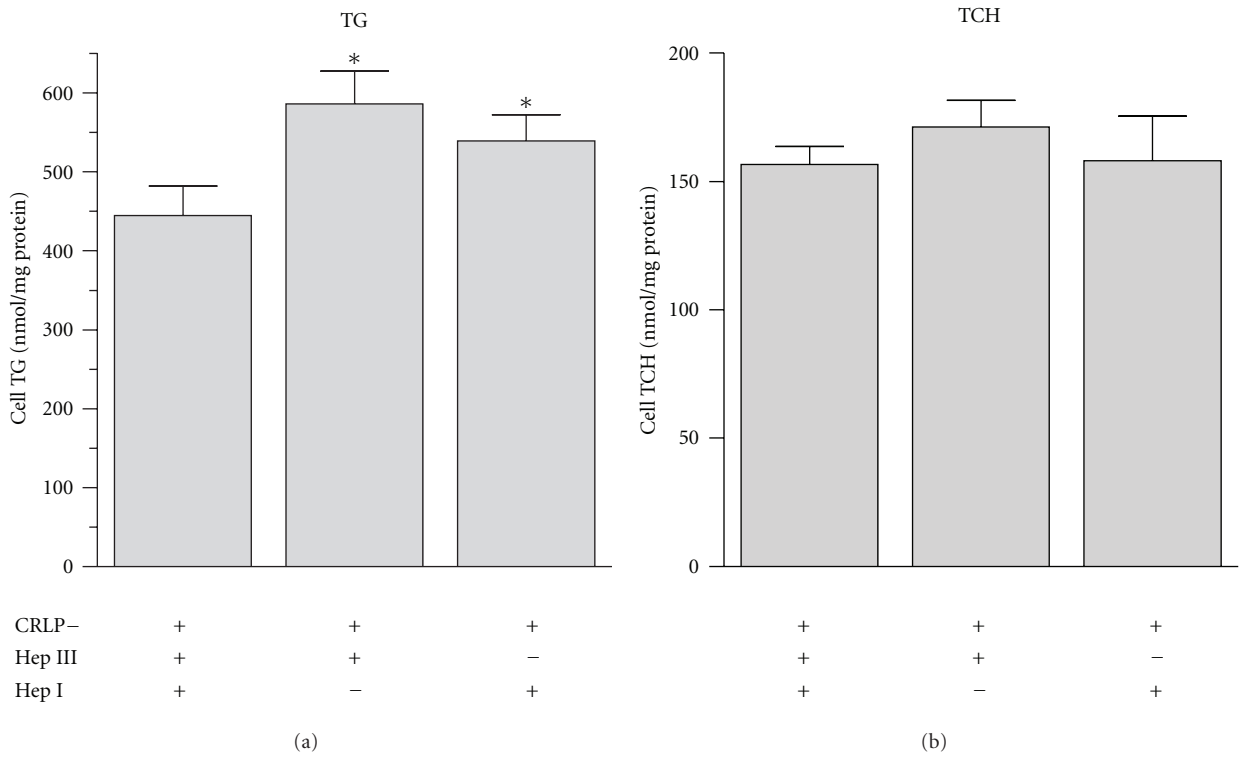


Figure 4: Role of proteoglycan-mediated bridging in macrophage lipid accumulation. HMDM were incubated 1 h in serum-free medium before treatment with 33 U/mL heparinase III (Hep III) or 33 U/mL heparinase I (Hep I) in combination with 50 mM Na chlorate. Both untreated and treated macrophages were then incubated 24 h with 80 μg cholesterol/mL CRLPw/o. Treatments were continued during the incubations. Macrophage triacylglycerol (TG) and total cholesterol (TCH) contents are reported as nmol/mg protein (means ± SD, $n = 4$). $^*P < 0.005$ versus control.

CRLPw/o particles. HMDM were incubated with [^{3}H]TG-CRLPw/o (80 μg cholesterol/mL) in the presence of different lipase inhibitors, and cell incorporation of CRLPw/o lipid was evaluated by measuring the radioactivity recovered in macrophage lipids. To inhibit cholesteryl ester hydrolase, LPL and sPLA$_2$, way121.989 [33], orlistat [34], and manoalide [35], respectively, were used. After incorporation into macrophages, radioactivity transported by [^{3}H]TG is redistributed into cellular fatty acids. To assess this, at the end of incubations with [^{3}H]TG-CRLPw/o, lipids were extracted from the cells and the radioactivity associated with TG, PL, free fatty acids, and cholesteryl esters was determined [7]. Most of the radioactivity was found in macrophage [^{3}H]TG and, much less, about 1/10, was in the [^{3}H]PL fraction. The radioactivity associated with free fatty acids and cholesteryl esters was negligible and was not taken into account in further analysis of the data. Thus, the total radioactivity taken up by macrophages was calculated as the sum of [^{3}H]TG + [^{3}H]PL. The results are shown in Figure 5. None of the inhibitors had any significant effect on the incorporation of radioactivity into PL (central panel), although all of them tended to decrease the total radioactivity taken up by macrophages, compared with macrophages incubated without inhibitors (control). However, the reductions were significant in comparison to the control only for manoalide ($P < 0.05$ and $P < 0.001$ at 0.2 and 2 μM of manoalide, respectively; $n = 3$) and for the higher concentration (2 μM) of orlistat ($P < 0.05$; $n = 3$). The decreased total radioactivity incorporated by macrophages treated with both concentrations of manoalide reflected the lower macrophage accumulation of [^{3}H]TG (upper panel) in these conditions, in comparison with either the control or way121.989. Thus, the internalization of TG carried by CRLPw/o is mainly dependent on sPLA$_2$ activity, but not cholesteryl ester hydrolase or LPL.

3.8. Role of Lipase Activities in TG and PL Synthesis after Uptake of CRLP by Macrophage. To assess how lipases affect lipid synthesis in HMDM after uptake of CRLPw/o, cells were preincubated for 2 h with or without inhibitors of sPLA$_2$ (MJ33 10 μM), cytosolic PLA$_2$ (MAFP, 5 μM and 10 μM) [36], calcium-dependent cytosolic PLA$_2$ (HELSS, 5 μM) [37] and LPL (orlistat, 2 μM). Incubation was then continued for 5 h in the presence of CRLPw/o (80 μg cholesterol/mL) to evaluate the incorporation of [^{3}H]glycerol into TG and PL (Figure 6).

PL synthesis was not significantly affected by any of the inhibitors tested (data not shown), but HELSS and MAFP at a concentration of 10 μM were found to inhibit TG synthesis ($P < 0.05$), while orlistat, MJ33, and 5 μM MAFP had no significant effect. These results suggest that cytosolic and calcium-dependent PLA$_2$, but not sPLA$_2$ or LPL, play a part

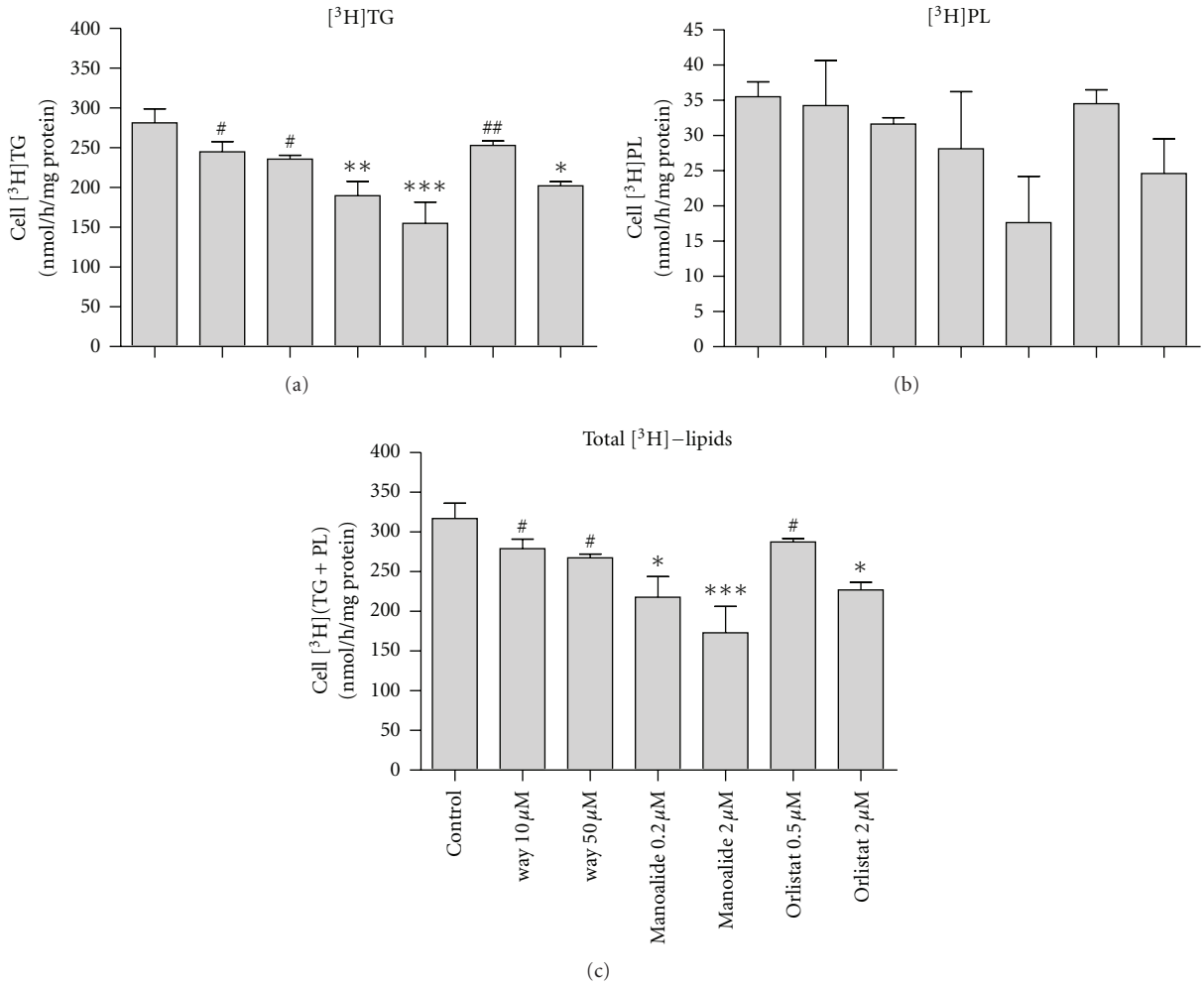


Figure 5: Role of macrophage-secreted lipases on [^{3}H]TG-CRLPw/o internalization. Cell radioactivity associated with macrophage lipids was determined after a 6-h incubation of HMDM with 80 μg cholesterol/mL of [^{3}H]TG-CRLPw/o in the presence of 10 and 30 μM way121.989 (way), 0.2 and 2 μM of manoalide, 0.2 and 2 μM of orlistat or in the absence of any inhibitor (control). Radioactivity associated with free fatty acids and cholesteryl ester was negligible, and that associated with macrophage triacylglycerol ([^{3}H]TG, (a)), phospholipid ([^{3}H]PL, (b)), and [^{3}H](TG + PL) (c) is reported as nmol/h/mg protein ($n = 3$). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus manoalide 2 μM.

in the regulation of TG synthesis after uptake of CRLPw/o by human macrophages.

4. Discussion

Chylomicron remnants can induce TG accumulation and foam cell formation [7–10]; however, the interactions between these postprandial lipoproteins and macrophages are poorly understood. Much evidence suggests that both receptor-dependent and -independent mechanisms function in lipid accumulation, as apolipoproteins may not be necessary for receptor interaction [6, 38] or induction of macrophage lipid accumulation [6, 12, 39]. However, studies and manipulation with chylomicron remnants are methodologically limited. There are marked difficulties in the preparation of postprandial lipoprotein fractions not contaminated by other lipoproteins. Thus, although studies performed with lipoprotein models devoid of apolipo-proteins may not reproduce physiological conditions, these models help to clarify the apolipoprotein receptor-independent mechanisms, which contribute in vivo to the accumulation of lipids in the vessel wall. We focused the current investigation on the mechanisms of internalization involved in lipid accumulation caused by apolipoprotein-free CRLPw/o. Macrophages are now known to have both pro- and anti-inflammatory properties; inflammation is essential for protection against pathogens, but healing requires the deleterious effects on the tissues to be suppressed. This dual role is facilitated by alternative activation of the cells into a pro- (M1) or anti-inflammatory (M2) phenotype [40]. Since our macrophage model is obtained in vitro in the absence of any stimulus, taking into account the limitations of a cell model, we believe it resembles more closely the classically polarized, round-shaped activated M1 macrophages.

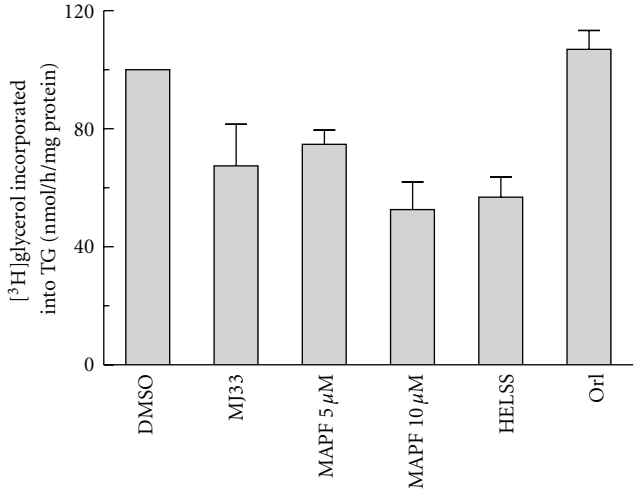


Figure 6: Role of macrophage lipase activities in TG synthesis. HMDM were preincubated for 2 h with or without 10 μM MJ33, 5 μM and 10 μM MAFP [36], 5 μM HELSS, and 2 μM orlistat. Incubation was then continued for 5 h in presence of CRLPw/o (80 μg cholesterol/mL) in the presence of [^{3}H]glycerol (4 μCi/mL, 20 μM) to evaluate the incorporation of [^{3}H]glycerol into TG. Radioactivity associated with triacylglycerol ([^{3}H]TG) is reported as nmol/h/mg protein ($n = 3$). $^*P < 0.05$ versus control.

Macrophages are now known to have both pro- and anti-inflammatory properties; inflammation is essential for protection against pathogens, but healing requires the deleterious effects on the tissues to be suppressed. This dual role is facilitated by alternative activation of the cells into a pro- (M1) or anti-inflammatory (M2) phenotype [40]. Since our macrophage model is obtained in vitro in the absence of any stimulus, taking into account the limitations of a cell model, we believe it resembles more closely the classically polarized, round-shaped activated M1 macrophages.

We found that the incubation of CRLPw/o with plasma induces some changes in compositions of these lipid particles including the acquisition of apolipoproteins [11], as well as the decrease of change of the ratios TG/TCH and FCH/TCH (Table 1). Despite their similar TG concentrations, CRLPw/o contain less TCH with a larger proportion of FCH (about 50%), which probably functions to stabilize the surface of these particles devoid of apolipoproteins. Thus, in comparison with CRLP+, CRLPw/o are larger buoyant particles (Figure 1), stabilized at their surface by a higher number of FCH molecules.

The lack of apolipoproteins on CRLPw/o did not prevent a significant increase of TG in both HMDM and J774 cell line macrophages during incubation with CRLPw/o. CRLPw/o induced TG accumulation independently of both phagocytosis and macropinocytosis. Macropinocytosis and phagocytosis are related but independent actin-dependent processes functioning in macrophages [19] and can contribute to foam cell formation by facilitating lipid accumulation. In particular, macropinocytosis, which functions in uptake of particles in the fluid phase, has been shown to mediate the induction of foam cell formation by LDL [20]. However, macropinocytosis is particularly efficient at high levels of lipoprotein that could not be achieved with CRLP in the current investigation due to experimental limitations. Chylomicron remnants are specialized to transport high quantities of TG, which at elevated concentrations induce cell detachment and toxicity [6, 41, 42]. We also found that CRLPw/o induce slight cell detachment at a concentration higher than 80 μg cholesterol/mL (data not shown). Thus, in our experimental conditions, conclusions about the lack of a role of macropinocytosis and phagocytosis involvement in CRLP processing are limited to the concentrations that we were able to test.

A possible mechanism for macrophage uptake of CRLPw/o is that they acquire macrophage-secreted apoE, which could lead to whole lipid particle uptake mediated by macrophage apoE-dependent receptors [43]. However, an anti-apoE antibody had no effect on TG accumulation induced by CRLPw/o in HMDM, suggesting that apoE is not necessary for CRLPw/o-mediated lipid accumulation in these cells and this conclusion was supported by the finding that CRLPw/o induced TG accumulation in J774 macrophages, a murine cell line which does not secrete apoE [24]. These results are in agreement with the observations of Fujioka et al. [6], who found that macrophages from apoE-deficient mice internalize apolipoprotein-free remnant particles.

LPL and PLA_2, as well as apoE secreted by macrophages, contribute to lipoprotein processing by acting as bridges between lipoproteins and heparan sulfate proteoglycans [25, 26]. However, our experiments exclude the possibility that extracellular lipases or other proteins that may act in this way are involved in macrophage CRLPw/o processing. Treatment with either heparinase I or heparinase III, which cause a loss of surface proteoglycans, increased CRLPw/o-induced macrophage TG accumulation (Figure 4), suggesting that surface proteoglycans may hinder rather than promote macrophage uptake of CRLPw/o.

The observation that the inhibition of macrophage secretion by brefeldin decreases CRLPw/o induced TG accumulation (Figure 3) focused our attention on macrophage secretory products. Although impairment of proteoglycans, apoE or LPL functions did not prevent TG accumulation, the inhibition of $sPLA_2$ markedly reduced the internalization of CRLPw/o, indicating a role for this enzyme in the metabolism of TG-rich lipoproteins. As the free fatty acids released after extracellular lipolysis enter cells and are re-esterified, contributing to an increase in cell TG content, we expected that the LPL inhibitor orlistat would cause a reduction in cellular TG accumulation induced by CRLPw/o. Surprisingly, in contrast to what has been reported for VLDL and chylomicron remnants [13, 29, 43], the experiments with radiolabelled particles showed that LPL is not a major factor involved in the uptake of [^{3}H]TG in CRLP devoid of apolipoproteins. The higher concentration of orlistat decreased the internalization of the [^{3}H]TG-CRLPw/o (−29%) only when the total lipid radioactivity was taken into account (Figure 5(c)), while the changes in macrophage [^{3}H]TG were not significant. On the other hand, the inhibition of $sPLA_2$ activity by manoalide reduced in the

incorporation of radioactivity into cellular [^{3}H]TG as well as the total lipid radioactivity in a dose-dependent manner. The inhibition of [^{3}H]TG-CRLP accumulation by HMDM induced by 0.5 μM and 2 μM of this inhibitor was about 32 and 45%, respectively, showing that, in contrast to LPL action, the catalytic activity of $sPLA_2$ has a prominent role in macrophage accumulation of TG during incubation with CRLPw/o.

This observation adds further details to the complex picture of the role of $sPLA_2$ in the development of cardiovascular disease. The current opinion is that circulating members of the PLA_2 family are positively associated with the pathogenesis of atherosclerosis by different mechanisms, including the generation of atherogenic particles [44], as well as the activation of several proinflammatory pathways [45]. Several physiological actions of $sPLA_2$ are unrelated to their enzymatic activity; rather, they can be attributed to the engagement of specific receptors on target cells [46]. However, in this study, the hypothesis that manoalide influences [^{3}H]TG-CRLPw/o metabolism by interfering with the specific binding of $sPLA_2$ to a surface receptor seems unlikely, because the manoalide binds irreversibly to several lysine residues of the enzyme, inhibiting specifically its activity [35]. Furthermore, the macrophage $sPLA_2$ subtypes comprise a diverse family of enzymes that catalyze the hydrolysis of the sn-2 ester bond of PL and glycerophospholipids but do not act on TG [31, 47, 48]. Thus, the effects of manoalide on cell [^{3}H]TG recovery, in contrast to LPL, cannot be explained in terms of massive extracellular TG hydrolysis followed by intracellular re-esterification. In addition, as the radioactivity was specifically carried by [^{3}H]TG in CRLPw/o, extracellular hydrolysis of PL did not contribute to the macrophage accumulation of [^{3}H]TG observed in our study. However, since the particles contain 70% TG and 25% PL (of which about 85% can be substrates of PLA_2), and TG contains 3 fatty acids that can be hydrolyzed and re-esterified, while PL contain only 2 fatty acids (with only 1 hydrolyzable by PLA_2), about 1/10 of the fatty that can be re-esterified are in the PL and 9/10 in the TG. Thus, the finding that, after uptake of the radiolabelled CRLPw/o, approximately 1/10 of the radioactivity is found in phospholipids suggests that the whole particles are taken up by the cells and that phospholipids are then hydrolyzed and re-esterified. Overall, our results indicate that $sPLA_2$ activity could contribute to CRLPw/o lipid internalization by inducing lipoprotein modifications which, in turn, increase the catabolism of the whole particle and/or selectively facilitate uptake of the TG moiety. For instance, Tietge et al. [49] reported that overexpression of $sPLA_2$ enzymatic activity alters the structure and composition of high-density lipoprotein (HDL) particles, enhancing selective uptake of cholesteryl ester with the metabolic consequences of increased catabolism of HDL. Similarly, some changes to CRLPw/o induced by the catalytic activity of $sPLA_2$ could expose or hide domains in the particles, which then could facilitate macrophage uptake of the TG carried by CRLPw/o. More studies are needed, however, to substantiate this idea.

Although our data indicate a role for $sPLA_2$ in the internalization of CRLPw/o, the inhibition of the enzyme did not affect the synthesis of TG from glycerol in HMDM after uptake of the particles (Figure 6). Interestingly, however, the results of this set of experiments indicate the involvement of cytoplasmatic PLA_2 and in particular of Ca2+-dependent PLA_2 in macrophage CRLPw/o metabolism. As MAFP is an irreversible inhibitor of of both calcium-dependent and calcium-independent cytosolic phospholipase A_2, but not secretory phospholipase A_2 [36], the finding that TG synthesis was decreased in the presence of this inhibitor supports the hypothesis that cytosolic PLA_2 activity plays a role in CRLPw/o processing. In particular, our results directly implicate the Ca2+-dependent PLA_2 activity, since HELSS, a direct and irreversible inhibitor of this enzyme [37], reduced the synthesis of TG induced by CRLPw/o in a similar manner to MAFP (Figure 6).

In conclusion, this study shows that $sPLA_2$ plays a role in the extensive macrophage TG accumulation promoted by TG-rich chylomicron remnant-like lipoproteins. Furthermore, this macrophage TG accumulation occurs independently of apolipoprotein-mediated receptor interactions, supporting the concept that more attention should be paid to pathways for macrophage lipid internalization not mediated by apolipoprotein-receptor interactions.

Abbreviations

apoE: Apolipoprotein E;
CRLP: Chylomicron remnant-like particles;
FCH: Free cholesterol;
HMDM: Human monocyte-derived macrophages;
IgG: Immunoglobulin G;
IMDM: Iscove's Dulbecco's modified medium;
LPL: Lipoprotein lipase;
PL: Phospholipid;
$sPLA_2$: Secretory phospholipase A2;
TCH: Total cholesterol;
TG: Triacylglycerol.

Acknowledgments

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