Atherosclerosis in the HIV and non-HIV setting: detecting and modifying cardiovascular risk

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Raaj Sankatsing is married to Vandana Mahadew and they have two children, a son named Karan, and a daughter named Shiranie.

Atherosclerosis in the HIV and Non-HIV setting: detecting and modifying cardiovascular risk

Raaj R. Sankatsing

UITNODIGING

Voor het bijwonen van de openbare verdediging van het proefschrift

Atherosclerosis in the HIV and Non-HIV setting: detecting and modifying cardiovascular risk

van Raaj Sankatsing

Woensdag 19 november 2008 om 12:00 uur in de aula van de Universiteit van Amsterdam: Oude Lutherse Kerk, Singel 417, te Amsterdam

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Na afloop in de Oude Lutherse Kerk

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Atherosclerosis in the HIV and Non-HIV setting: detecting and modifying cardiovascular risk

Aan mijn ouders
Atherosclerosis in the HIV and Non-HIV setting: detecting and modifying cardiovascular risk

ACADEMISCH PROEFSCHRIFT

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in het openbaar te verdedigen in de Aula der Universiteit
op woensdag 19 november 2008, te 12.00 uur

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Raaj Ramakant Sankatsing

geboren te Paramaribo, Suriname
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General introduction
General introduction

Cardiovascular disease (CVD) poses one of the most deadliest threats to western societies and will soon become a global epidemic as the incidence is rising rapidly both in the developed and developing world. At the core of CVD lies a process which is called atherosclerosis. Atherosclerosis is a progressive disease and is characterized by the gradual accumulation of lipids and fibrous material in large arteries [1]. The development of atherosclerosis starts at an early age and has a multifactorial origin. Familial hypercholesterolemia, familial combined hyperlipidemia and familial hypoalphalipoproteinemia are some of the genetic disorders in lipoprotein metabolism which are associated with the atherosclerosis process. Traditional risk factors contributing to the development of atherosclerosis include hypercholesterolemia, smoking, hypertension, diabetes mellitus, obesity and a sedentary lifestyle [2]. Treatment and prevention of atherosclerosis has been the subject of intensive research during the last decades. Statins, which lower low-density lipoprotein cholesterol (LDL-C) by inhibiting hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase, are currently the single most important drug class used to slow this process of atherosclerosis.

Recent studies have shown that some unrelated medical conditions are also associated with an increased risk of developing CVD. These include, but are not limited to, disorders such as rheumatoid arthritis, systemic lupus erythematosus, antiphospholipid syndrome [3] and human immunodeficiency virus (HIV) infection [4]. HIV infection has indeed been associated with acceleration of atherosclerosis, either by itself or through its effects on the immune system resulting in chronic immune and coagulation activation.

Ironically, effectively treating these HIV-infected patients with antiretroviral therapy greatly improves HIV-related morbidity and mortality but also seems to increase the incidence of CVD [4]. This increased risk of CVD initially seemed to be confined to those patients treated with protease inhibitors [5], but more recently agents belonging to the class of nucleoside reverse transcriptase inhibitors, particularly abacavir, were also reported to be associated with increased CVD risk [6]. The risk associated with HIV protease inhibitors is plausible given the fact that protease inhibitors are known to increase plasma levels of LDL-C and triglycerides. These lipid particles are highly atherogenic. LDL-C is one of the key components contributing to the development of an atherosclerotic plaque within the vessel wall. Abacavir in contrast has been hypothesized to exert its risk by promoting inflammation. Thus, HIV-infected patients are currently surviving longer as a result of the available effective antiretroviral therapy, but are also confronted with an increased incidence of cardiovascular disease. At present, approximately 33 million people are infected with HIV worldwide [7] and although only a small proportion of these people are currently receiving effective antiretroviral treatment it is expected that the number of treated patients will rise steadily in the coming years, partly because of an in-
increased availability of low-cost antiretroviral drugs, including generics produced in developing countries themselves. The increasing number of treated patients will also lead to more patients developing dyslipidemia and requiring further treatment. How can this be dealt with effectively and more importantly, can we do anything to prevent the potential epidemic of CVD awaiting HIV-infected patients worldwide? In this thesis, we present current knowledge about the determinants of this new phenomenon in HIV-infected patients and also discuss possible treatment modalities as well as provide suggestions as to how to decrease the risk of developing CVD.

Outline of the thesis

In the first part we review the current state of surrogate markers for cardiovascular disease in general. Atherosclerosis progresses slowly over many years before becoming clinically manifest as acute cardiovascular events late in the disease process. Studies examining pharmaceutical interventions therefore require long-term follow up in large populations in order to determine clinical benefit of such interventions. In order to avoid these time consuming and expensive intervention studies, the use of surrogate markers have gained wide interest in the last decade. In chapter 1 the existing cardiovascular imaging modalities, both invasive and non-invasive, are discussed. In chapter 2 we focus on carotid intima-media thickness, a non-invasive surrogate marker, and discuss its applicability in both observational as well as in drug intervention trials.

In the second part we focus on the role of low-density lipoprotein cholesterol (LDL-C) in atherosclerosis. To substantiate the impact of LDL-C lowering, we addressed surrogate markers for atherogenesis in a group of patients characterized by genetically-determined low LDL-C levels due to a mutation in apolipoprotein B (apoB), the major protein within the LDL-C fraction: familial hypobetalipoproteinemia (FHBL). In chapter 3 we report the results of direct sequencing of the entire apoB gene in order to identify the cause of the extremely low levels of ApoB and LDL-C in subjects with FHBL. Using these same FHBL subjects we investigated the effects of extremely low levels of LDL-C on surrogate markers for atherogenesis by evaluating both carotid intima-media thickness and arterial stiffness in FHBL subjects and compared these with observations in healthy controls (chapter 4).

The majority of cardiovascular events are not prevented despite the widespread use of (high dose) statins. Recent clinical trials have raised the awareness for aggressive lowering of LDL-C showing additional clinical benefit when LDL-C goals are lowered to below 100 mcg/dL (2.6 mmol/L) [8, 9]. In chapter 5 we review the clinical experience of a novel drug which aggressively lowers LDL-C by inhibiting both cholesterol production as well as intestinal cholesterol absorption. Both efficacy as well as safety issues are discussed.
In the third part of the thesis we address the cardiovascular consequences of HIV infection. The cardiovascular risk associated with HIV infection, its treatment and possible therapeutic options are discussed.

In chapter 6 we review the available evidence in the literature linking HIV infection and its treatment with atherosclerosis and summarize the multiple mechanisms by which HIV-infected patients are at increased risk of developing cardiovascular disease. In chapter 7 we investigate the differential effects of two antiretroviral drugs on lipids and lipoproteins in neonates born to HIV-infected mothers, who have received this treatment in order to prevent transmission of HIV from mother to child. By specifically evaluating these drugs in HIV uninfected newborns, this allows for accurate evaluation of the lipid-consequences of these drugs in absence of confounding factors such as chronic inflammation.

Chapter 8. In the HIV-field, there still exists a lot of debate whether and to what extent accelerated atherogenesis can be attributed to all drugs used to treat HIV or only a subgroup of drugs (mostly protease inhibitors). To address this issue in more detail, we compared the effects on lipids and carotid intima media thickness of chronic, stable use of protease-inhibitors versus non-nucleoside reverse transcriptase inhibitors (NNRTIs). Thus, we confirmed a clear HDL-C increase in patients using NNRTIs, the mechanism of which is as yet unclear. In chapter 9 we addressed the mechanism by which Nevirapine, one of the NNRTIs, increases HDL-C. However, in order to establish optimal lowering of cardiovascular risk in HIV-1 infected patients, potent statin therapy remains treatment of first choice. However, statin therapy is often limited or even omitted, partly due to the fact that interaction between HIV-treatment drugs and statins are feared. In chapter 10 the results of a pilot study are presented in which the efficacy and safety of rosuvastatin, a statin which would be expected not to have any interaction with protease inhibitors, was investigated when used to treat dyslipidemia caused by the protease inhibitor lopinavir/ritonavir (Kaletra®).
Reference List

Part I

Imaging of (sub)clinical atherosclerosis
Abstract

Purpose of review
Novel treatment modalities for cardiovascular prevention are emerging rapidly. Since it is virtually impossible to evaluate all these new compounds in long-term trials using clinical endpoints, there is an urgent need for validated surrogate markers of atherosclerosis in order to save both time and costs. Over the last decade, particularly the use of imaging markers has been widely introduced into drug development strategies. In the present review we will discuss the most commonly used techniques.

Recent findings
Whereas both testing of endothelial function assessed as flow mediated dilation and assessment of carotid intima–media thickness have been shown to predict future cardiovascular events, predominantly IMT has been successfully used as surrogate marker in intervention studies. More recently, standardization of intravascular ultrasound has also enabled reproducible assessment of coronary atheroma volume. Multi-slice computed tomography and electron beam computed tomography have proven useful in providing quantitative information on plaque burden and coronary calcium content, respectively. Although cardiovascular magnetic resonance (CMR) is continuously improving, additional technical improvements will be mandatory before this technique can be implemented in multicenter clinical studies.

Summary
The imaging modalities reviewed in this paper all provide specific information on either functionality of morphology of the vasculature. To date, the value of carotid IMT for cardiovascular risk prediction has been studied most extensively. Whereas assessment of plaque burden using IVUS appears to be the most direct way to quantify coronary changes, its predictive value for future cardiovascular events remains to be established. Awaiting further technical improvements, CMR is expected to provide the most valuable information for both quantitative and qualitative evaluation of atherosclerosis in the near future.
Introduction

Atherosclerosis is an inflammatory process that causes lesions in both large- and medium-sized arteries. The disease slowly progresses over many decades, causing clinical manifestations only at advanced stages of the atherosclerotic process. Due to this protracted time course, cardiovascular prevention trials using clinical endpoints usually require long-term follow-up and a large number of participants in order to be able to demonstrate clinical benefit of pharmaceutical interventions [1]. The corresponding studies are both expensive and time consuming [2]. One of the consequences is that innovative drugs may be withheld from patients for many years, awaiting final proof of efficacy [3]. To circumvent these issues, surrogate markers are increasingly being used in cardiovascular intervention studies [2]. This review addresses the available imaging modalities of both the arterial wall as well as the arterial lumen which are currently being used as surrogate markers.
Figure 1. Flow-Mediated Dilatation

Image frame of automated measurements of the brachial arterial lumen and the associated flow mediated dilatation curve (Brachial Analyzer, MIA vascular tools, Coralville USA). Y-axis: brachial lumen diameter (mm); X-axis: image frame numbers (obtained every third heart beat. Brachial FMD in this example is \((4.62 - 4.34)/4.34 \times 100 = 6.5\%\).

Figure 2. B-mode Intima-Media Thickness

High resolution 15MHz B-mode ultrasound image of the distal common carotid artery far wall in a healthy 23 year old volunteer. The vertical arrow indicates the carotid dilatation, just proximal of the carotid bulb. Intima-media thickness (IMT) is defined as the distance between the lumen-intima and the media-adventitia interfaces (upper and lower red lines). Measurements are done along a 10mm length of the wall. The average IMT of this particular wall was 0.049 (SD 0.008) mm.
Non-invasive and invasive imaging of atherosclerosis

Flow Mediated Dilatation

The crucial role of the vascular endothelium as first line defense mechanism against atherogenic insults has been generally acknowledged. All known cardiovascular risk factors, such as hypertension [4], smoking [5], hypercholesterolemia and diabetes mellitus [6] have been shown to contribute to onset of endothelial dysfunction. In line, endothelial dysfunction is one of the earliest stages of atherogenesis, preceding the occurrence of atherosclerotic lesion formation [7]. More recently, endothelial dysfunction has also been shown to have predictive value for cardiovascular events [8;9]. Besides invasive techniques to measure endothelial function using intra-arterial infusion of selective endothelial agonists, the introduction of flow mediated dilation to test endothelial function has paved the way for wider application [10;11]. The basic principle of this measurement pertains to the induction of increased blood flow in the brachial artery, following deflation of an occluding forearm cuff. The ensuing reactive hyperemia causes increased shear stress at the level of the endothelium of the brachial artery, which induces production of nitric oxide (NO) resulting in relaxation of vascular smooth muscle cells [12]. The ensuing diameter increase of the brachial artery can be measured using ultrasound diameter measurements. The advantages of this technique are its non-invasive and readily applicable nature. However, substantial variation in reproducibility has been described, due to both differences in technical protocols as well as the impact of physiological factors on FMD [10]. Careful standardization of the protocol, including the implementation of automated real-time vessel boundary detection, has contributed significantly to reduce the variability (Figure 1). Yet, FMD values in healthy middle-aged subjects may still vary extensively, ranging from 5% to 21% [13]. The large interindividual variation limits the use of FMD as an individual cardiovascular risk marker. In spite of these shortcomings, FMD has proven to be a valuable parameter to evaluate changes in endothelial function at a group level [14].

B-mode ultrasound carotid intima-media thickness

High-resolution B-mode ultrasonography of the carotid arterial far-wall enables visualization of the distance between the lumen-intima interface and media-adventitia interface, reflecting the carotid intima-media thickness (CIMT) (Figure 2). This non-invasive technique allows for real-time in vivo imaging of all stages of atherosclerosis, going from the normal arterial wall to complete arterial occlusion.

Accordingly, arterial wall thickness can be measured as a continuous variable from childhood to old age, in patients as well as in healthy controls [15]. The great variability in measurement
protocols which could affect clinical trial outcomes obviates the need for standardization of these protocols in order to permit reliable comparison of study results. Nevertheless, under standardized conditions, the technique of CIMT offers good reproducibility. This makes it suitable to apply in relatively small, comparative studies investigating vascular pathophysiology, as well as in large, multicenter clinical trials.

Three large observational studies utilizing IMT as a surrogate marker for CVD are the Atherosclerosis Risk in Communities (ARIC) Study [16], the Cardiovascular Health Study (CHS) [17], and the Rotterdam Study [18;19]. In the ARIC study (n=12841), IMT was able to assess all stages of atherosclerosis and proved an independent predictor of coronary artery disease. These findings could be confirmed in the subsequent CHS (n= 5858) and the Rotterdam Study (n=8000). In addition, statin intervention trials such as ASAP [20], REGRESS [21;22] and ARBITER-I [23], have underscored the value of CIMT as an efficient parameter to assess efficacy of lipid treatment. Both ASAP and ARBITER-I showed that aggressive lipid lowering with 80 mg of atorvastatin was associated with a decrease in carotid IMT as opposed to no change or progression in the comparative low-dose statin arms.

Various studies have shown the strong correlation between IMT with cardiovascular risk factors such as LDL-C [24-27], HDL-C [28;29] and blood pressure [30]. Collectively, carotid IMT has proven to be a well standardized and validated surrogate marker for CVD and is particularly closely correlated with CAD and incidence of cardiovascular events such as myocardial infarction and stroke [17;18;31-33].

Quantitative coronary angiography

In patients with coronary atherosclerosis, angiographically determined progression of the disease is one of the major factors determining clinical prognosis[34-36]. This makes serial angiography a ‘validated’ technique as surrogate marker for cardiovascular risk, which has been used successfully used in many multicenter trials [21;36]. Coronary atherosclerosis is a complex process that is not limited to focal areas of the coronary artery tree [37]. Therefore, to assess the effect of an intervention on progression or regression of coronary atherosclerosis, both focal (minimal lumen/obstruction diameter in mm) and diffuse (mean lumen diameter) changes should be measured [36]. Visual interpretation of coronary angiograms has its limitations because assessment of stenosis severity is associated with: a) a large intra- and inter-observer grading variability (8-37%) b) only relative stenosis measurements are provided and c) severity of diffuse atherosclerosis is difficult to estimate. Therefore, Quantitative Coronary Angiography (QCA) providing absolute diameters in mm is now the established standard [38] (Figure 3).

Of note, early stages of coronary atherosclerosis are associated with remodeling of the coronary artery [39], resulting in preservation of the lumen cross sectional area. Consequently, early stage coronary atherosclerosis is angiographically undetectable [40]. This often causes underestimation of the severity and extent of coronary atherosclerosis when compared to
Surrogate markers for atherosclerotic disease post-mortem pathological studies [41]. Despite the limitations, standardized QCA has proven itself as a worthwhile technique, carrying important prognostic information.

Figure 3. Quantitive Coronary Angiography

![An example of a coronary bifurcation analysis with QVA-CMS® V6.0](image)

The bifurcation analysis output shows the arterial contours of the proximal and two distal artery segments of the bifurcation as one. For each of the three artery segments (each including its central part of the bifurcation) separate reference contours and reference diameter functions (graphs) are created. Some major advantages of this technique are: 1) A proper determination of the reference diameter function for each individual artery segment in combination with its central part of the bifurcation, resulting in the correct estimation of the reference diameter and percentage diameter stenosis. 2) A proper determination of the obstruction length (see Distal) and better positioning of the obstruction marker when the lesion is overlapping with or close to the central part of the bifurcation. This is due to the presence of the elongated arterial- and reference contours.

**CT coronary imaging**

Non-invasive visualization of the coronary arteries puts any diagnostic technique to the test, because the coronaries are small, tortuous, and respiratory motion and the continuous cardiac motion distorts the image. Thus, high temporal resolution is required to “freeze” the heart to produce a sharp image. Despite these hurdles CT (Computed Tomography)-scanners are able to visualize the coronary arteries.
**Electron Beam Computed Tomography**

Electron-beam Computed Tomography (EBCT) can accurately and reproducibly quantify the presence of coronary calcium in the coronary tree. Whereas the ‘coronary calcification score’ correlates well with total atherosclerotic plaque burden, it only reflects the presence of advanced lesions. Several studies have shown that coronary artery calcium (CAC) is a marker of increased risk for adverse coronary events[42], indepently from other CV risk factors [43;44]. To date, only few studies are available, reporting decreased progression of CAC during active treatment [45].

**Multidetector CT**

Multidetector CT coronary imaging represents cross-sectional imaging of the coronary lumen and wall which allows quantification of coronary plaques as well as the artery wall[46;47]. In addition, valuable information with respect to the composition of the plaque can be obtained based on the differences of the CT-density values [48;49] (Figure 4).Based on the presence or distribution of plaques (1,2,3 vessel disease) and stenosis severity (> 50% diameter stenosis) one can establish the plaque burden of the coronary tree [50]. However, it remains to be established whether the CT-assessment of the coronary plaque burden is accurate in stratifying cardiac risk. In addition, further studies are needed to further explore the ability of MD-CT to assess disease progression, stabilization or even regression following specific therapy.

**Figure 4. MSCT Coronary Plaque Imaging**

Atheroma Calcific plaque Fibrotic plaque Complicated plaque with thrombus

Low density HU 30 High density HU 480 Intermediate density HU 85 Very low density HU 10

The density values expressed as Hounsfield Units (HU) are shown for atheroma, calcific plaque, fibrotic plaque and a plaque complicated by thrombus formation.

Shown are the axial images and for each lesion a cross-sectional image (inlays).
Cardiovascular magnetic resonance

Cardiovascular magnetic resonance (CMR) is a relatively new technique in the assessment of atherosclerosis. New sequences allow the vessel wall to be imaged such that either its area (2D technique) or volume (3D technique) can be measured and followed over time (Figure 5). The 3D technique has the merit of being less dependent on slice positioning in longitudinal studies, and is a more sensitive test, because the area of many slices is added in order to generate a vessel wall volume [51]. Most work has been performed in the carotid artery because it can be imaged in high resolution as it is near the surface. The 3D technique samples about 3cm either side of the carotid bifurcation and has an interstudy reproducibility of 4.4% [52]. The vessel wall volume is the difference between the external wall volume and the luminal volume, and semi-automated software is now available to make these measurements reliably and quickly (Atheroma-Tools, CVIS, London, UK).

A number of studies in humans have assessed CMR in the longitudinal measurement of atheroma. Mohiaddin showed atheroma progression in untreated aortic plaque area over 2 years [53]. Corti showed that simvastatin caused regression of aortic and carotid artery atheroma area over 12 [54], and 24 months [55]. Lima showed that simvastatin could induce regression of aortic plaque volume after only 6 months of treatment [56]. Yonemura showed regression of aortic plaque area after 12 months of simvastatin treatment [57]. In conclusion, atheroma CMR is beginning to be used for longitudinal follow-up of patients to investigate atheroma progression and regression. The good reproducibility has allowed current trials to be conducted in small samples sizes (~40 patients) over reasonable time periods (12 months) to show statistically significant effects.

Intravascular Ultrasound

Intravascular ultrasound (IVUS) is performed during cardiac catheterization using small, intracoronary catheters. The strong ultrasound signal reflected from the intima and external elastic membrane (EEM) allows real-time intraluminal imaging of the vessel wall and measurement of the atheroma (intima-media area) [58;59]. Initial quantitative IVUS studies examined the progression of plaque area at diseased lesion sites [60]. However, the reproducibility of this analysis approach was limited by the difficulties to exactly match individual sites. Accordingly volumetric analysis approaches integrate consecutive plaque area measurements at 0.5-1mm intervals along long vessel segments (Figure 6). Because the segment rather than individual sites are matched at baseline and follow-up, assessment of small percent changes in atheroma volume is possible with considerable statistical power [61-66]. In a recent randomized trial, intraobserver variability was analyzed in 1177 images from 18 patients [64]. The mean [SD] differences were negligible for both EEM (−0.16 [0.68] mm²) and lumen areas (−0.02 [0.75] mm²).
3D reconstruction of CMR of a carotid artery on the left for a normal subject, and on the right a patient with carotid artery atherosclerosis. The inner and outer borders of the carotid artery have been analysed semi-automatically with specialized software (Atheroma-Tools, www.CMRtools.com) and the total wall volume has been calculated from the difference. This is a sensitive measure of atherosclerosis burden which is highly reproducible allowing longitudinal changes in atheroma to be followed, especially with drug interventions, in small sample sizes. The total wall volume for the normal subject (left) is considerably lower than that of the patient with carotid atherosclerosis (right) and the arrows indicate a particular area of wall thickening at the carotid bulb.
Linear regression analysis showed close correlations between the original and re-analysis ($r = 0.99$ and $0.98$ for EEM and lumen areas). Interobserver variability was evaluated in 2151 images from 30 patients. The mean [SD] differences were negligible for both EEM ($-0.07 [0.93]$ mm$^2$) and lumen areas ($-0.07 [0.93]$ mm$^2$). Regression analysis showed close correlations between the original and re-analysis ($r = 0.99$ and $0.98$ for EEM and lumen areas).

Results from randomized, serial IVUS studies collectively demonstrate either arrest of progression or actual regression during intensive lipid-modifying therapies [61-66]. Sub-studies have also demonstrated the independent role of the inflammatory marker CRP [67]. The comparison of these IVUS trials with outcome studies using similar pharmacological interventions provide indirect data correlating plaque burden to clinical outcomes [64;67-69]. Collectively, these studies underscore the validity of volumetric plaque burden as endpoint for the assessment of atherosclerosis progression/regression.

Figure 6. Intravascular Ultrasound

For volumetric analysis of plaque burden, a segment of interest is selected between two characteristic fiducial points. Evenly spaced frames are selected at 0.5-1.0 mm intervals. For each of the selected frames, the lumen and EEM cross sectional areas are measured. The Simpson equation is applied to calculate plaque volume by multiplying plaque area and distance between adjacent images.

Conclusion

At present, the use of IMT as surrogate marker has several advantages compared to other imaging techniques: it is easy to use, non-invasive, has a good reproducibility and has the capacity to identify atherosclerosis progression and regression. At the same time, the validity of the method is supported by a large number of clinical trials. Whereas FMD also has predictive value for future CVD, this method is of limited use due to a large interindividual variation. Coronary angiography has traditionally been the ‘gold-standard’ imaging modality to assess coronary atherosclerosis progression and regression. However, this technique identifies only the arterial lumen and fails to identify early stages of the disease. Electron beam CT reliably quantifies calcium content in the coronary arteries. However, EBCT solely identifies advanced plaques. Cardiovascular MR has rapidly evolved as a means to assess carotid and coronary plaque area and volume. Further data are awaited to evaluate its reliability as a surrogate marker for CAD. IVUS permits assessment of plaque burden in vivo. Whereas changes in plaque burden as measured by IVUS may well be the most direct way to evaluate the effect of an anti-atherosclerotic agent, its predictive value for future CV events remains to be established.

In summary, ultrasound IMT of the carotid arteries is considered the predominant non-invasive surrogate marker for CVD, albeit in a non-coronary vessel, whereas IVUS is the preferred tool for invasive assessment of atherosclerotic disease where it is most relevant, i.e. in the coronary arteries.
References


Atherosclerosis measured by B-mode ultrasonography: effect of statin therapy on disease progression

Kastelein, J.J.
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Abstract

Changes in intima-media thickness (IMT) and arterial lumen diameter – as measured by B-mode high-resolution ultrasonography and quantitative coronary angiography, respectively – are currently the only surrogate markers for progression of atherosclerotic disease recognized by regulatory authorities in the United States and Europe. Because atherosclerosis is a disease of the arterial wall, the ability of B-mode ultrasonography to provide visualization of IMT offers significant advantages over angiography. These advantages, as well as the safety and noninvasive-ness of B-mode ultrasonography, have led to increasing use of this imaging technique in observational studies and interventional studies of lipid-lowering agents over the last decade. These observational studies clearly demonstrated an association between carotid IMT and atherosclerotic disease. Of the interventional studies, the recent Arterial Biology for the Investigation of the Treatment Effects of Reducing Cholesterol (ARBITER) trial found that use of atorvastatin 80 mg daily for aggressive lowering of plasma low-density lipoprotein cholesterol (LDL-C) concentrations to below current target levels was associated with significant IMT regression compared with results obtained with less aggressive plasma LDL-C lowering. A new study – Measuring Effects on Intima Media Thickness: an Evaluation of Rosuvastatin (METEOR) – will examine the effects of aggressive lipid-lowering treatment with rosuvastatin 40 mg daily on IMT. The cohort in this study will be individuals with mild hypercholesterolemia whose standard risk assessment does not categorize them as at sufficient risk of clinical disease to warrant initiation of lipid-lowering therapy despite their relatively high IMT values.
Atherosclerosis is a dynamic disease process characterized by vessel wall remodeling that occurs over decades, ultimately becoming clinically manifest as acute cardiovascular events in many individuals. Epidemiologic and interventional studies using cardiovascular clinical end points require long study periods and large populations to establish the influence of risk factors and effects of therapeutic interventions in preventing such disease outcomes. B-mode (2-dimensional) ultrasonographic imaging permits noninvasive, real-time, high-resolution imaging of superficial artery walls, thus allowing visualization of the effects of atherosclerotic processes on the vessel wall at every stage from relative absence of disease to complete arterial occlusion. Because atherosclerosis is a disease of the arterial wall, the ability of B-mode ultrasonography to provide visualization of carotid intima-media thickness (IMT) offers significant advantages over angiography. In particular, B-mode ultrasonography permits visualization of the entire artery wall at all stages of disease progression, whereas angiography permits visualization of the lumen only and detects changes only at very late stages in the disease process. Moreover, B-mode ultrasonography is safe and noninvasive, and so it can be used in observational studies of healthy patients as well as in atherosclerosis regression trials. Several questions about B-mode ultrasonography remain unanswered, including the most appropriate vessel for measurement of IMT (e.g., internal carotid artery vs. common carotid artery), its applicability in younger patients, and standardization of imaging protocols. Overall, however, the weight of evidence strongly supports the use of B-mode ultrasonography as a research tool in clinical trials of lipid-lowering agents: it allows disease progression and interventional effects to be assessed in smaller patient populations and over relatively shorter periods than in studies using clinical end points. To date, changes in IMT measured by B-mode high-resolution ultrasonography and changes in arterial lumen diameter determined by quantitative coronary angiography are the only surrogate markers for progression of atherosclerotic disease recognized by the US Food and Drug Administration (FDA).

This article reviews evidence from observational studies demonstrating that carotid IMT is increased in patients with established clinical cardiovascular disease (CVD) and that increased IMT is predictive of new-onset clinical disease. An overview of key 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor (statin) interventional trials using B-mode ultrasonography is provided, and the implications of these studies for the treatment of patients with various risk profiles, including those at low risk of experiencing clinical events but with relatively high IMT values, are discussed. A more nuanced understanding of patient risk is particularly important in light of 2 recent reports: the final report of the third Adult Treatment Panel (ATPIII) of the US National Cholesterol Education Program (NCEP), which significantly revised the use of Framingham risk assessment and increased the number of patients eligible for plasma lipid lowering therapy; and the UK Medical Research Council/ British Heart Foundation (MRC/BHF) Heart Protection Study (HPS), which demonstrated that statin treatment may benefit a wider range of individuals than previously believed.
Observational intima-media thickness studies

Several observational studies have shown that individuals with CVD have greater IMT values and that greater IMT is associated with increased risk of clinical disease. In an early study, Geroulakos et al. compared 75 patients undergoing coronary angiography for symptomatic coronary artery disease (CAD) with 40 asymptomatic matched control subjects. These investigators found that common carotid IMT was greater in patients than in control subjects and was greater in patients with stenosis on coronary angiography than in those with “normal” angiograms.

Two large studies that assessed the association of IMT on B-mode ultrasonography and atherosclerotic disease in the general community are the Atherosclerosis Risk in Communities (ARIC) Study and the Rotterdam Study. In the ARIC Study, assessment of the far wall of the carotid artery in 13,870 black and white men and women revealed that the mean carotid IMT was greater in individuals with prevalent CVD than in disease-free individuals across all race and sex strata. A mean carotid IMT increase of 0.2 mm increased the relative risk of myocardial infarction (MI) by 33% and stroke by 28%, suggesting that B-mode ultrasonography is a noninvasive predictor of CAD. Similar results were demonstrated by the Rotterdam Study, a single-center prospective study in 8,000 individuals >55 years in a suburb of Rotterdam: documented associations between carotid IMT and MI, stroke, angina, intermittent claudication, and hypertension. Over a mean 2.7 years of follow-up, a case-control substudy in subjects with or without MI or stroke showed that a higher baseline IMT was associated with increased risk of MI (odds ratio[OR], 1.43 per baseline IMT SD increment of 0.163) and that it increased the risk of stroke (OR, 1.41/SD increment). Greater risk of MI (OR, 1.51/SD increment) and stroke (OR, 1.57/SD increment) also was associated with higher baseline IMT in those subjects without a history of MI or stroke.

The association between IMT and CVD in asymptomatic patients was also seen in a study by the Cardiovascular Health Study Collaborative Research Group, which observed 4,476 patients without clinical CVD for a median follow-up of >6 years. Cumulative (unadjusted) cardiovascular event rates correlated with baseline carotid IMT quintile, with the rate of MI or stroke <5% in the lowest (first) quintile and >25% in the highest (fifth) quintile. The significant relation remained after adjustment for other risk factors; relative risk of MI or stroke increased to 1.54 in the second IMT quintile, 1.84 in the third quintile, 2.01 in the fourth quintile, and 3.15 in the fifth quintile.

These observational studies clearly demonstrate an association between carotid IMT and atherosclerotic disease. However, because carotid IMT is also strongly related to conventional risk factors, whether use of this biomarker improves the ability to stratify patients into high- and low-risk groups remains a matter of debate.
Statin interventional studies

The effects of statin therapy on atherosclerosis have been assessed by high-resolution ultrasonographic measurement of carotid IMT in a number of studies. It is important to keep in mind, however, that lack of a standardized protocol for measuring IMT change makes inter-study comparisons difficult. In particular, imaging protocols vary with respect to the carotid artery selected for IMT measurement, i.e., left or right common carotid artery, the carotid bulb, or the internal carotid artery, as well as the specific segment of the artery, i.e., near or far wall. Some trials have included both internal and common carotid arteries as outcome measures, whereas others have focused on the far wall of the common carotid artery.  

An example of variation due to the arterial segment selected occurred in the Asymptomatic Carotid Artery Progression Study (ACAPS)15: 919 asymptomatic men and women (aged 40 to 79 years) with early carotid atherosclerosis received lovastatin 20 to 40 mg daily or placebo (all received aspirin) and had carotid IMT measured at 6-month intervals for 3 years. The mean values for maximum carotid IMT decreased by 0.009 mm/yr in the lovastatin group and increased by 0.006 mm/yr in the placebo group (P = 0.001) (Figure 1). Plasma levels of low-density lipoprotein cholesterol (LDL-C) were reduced by 28% in the lovastatin recipients (from 157 to 113 mg/dL) and were unchanged in the placebo group. However, it is instructive to note that the results for ACAPS would have been negative if only far-wall common carotid artery IMT had been used as an outcome measure, illustrating the difficulty in making comparisons with studies that do not include IMT of the internal carotid artery as an outcome measure.6

Figure 1 Change in mean values for maximum carotid intima-media thickness (IMT) measurements in patients receiving either lovastatin 20 to 40 mg or placebo daily in the Asymptomatic Carotid Artery Progression Study (ACAPS). (Reprinted with permission from Circulation.15)
One such study is the Kuopio Atherosclerosis Prevention Study,\textsuperscript{16} which measured IMT in the right and left distal common carotid artery and the right and left carotid bulb but not in the internal carotid artery; 447 men (aged 44 to 65 years) with plasma levels of LDL-C $\geq 4.0$ mmol/L (154 mg/dL) and total cholesterol $< 7.5$ mmol/L (290 mg/dL) received pravastatin 40 mg or placebo daily for 3 years. Overall mean carotid IMT increased by 0.017 mm/yr in the pravastatin group and increased by 0.031 mm/yr in the placebo group ($P < 0.005$). Plasma LDL-C concentration was reduced by 27\% in the pravastatin group and remained unchanged in the placebo group. The study investigators suggested that the straight segment of the common carotid artery be used as the site for B-mode ultrasonography in future studies assessing atherosclerotic disease progression.

The importance of common carotid artery measurements was also demonstrated in the Monitored Atherosclerosis Regression Study (MARS).\textsuperscript{17} Distal common carotid IMT was measured at 6-month intervals for $\leq 4$ years in 188 adults (aged 37 to 67 years) with angiographic CAD and a plasma total cholesterol value of 190 to 295 mg/dL who received either placebo plus dietary therapy or lovastatin 80 mg daily. IMT was significantly reduced in the lovastatin recipients as early as 1 year after initiation of therapy (Figure 2). Mean reductions in IMT were 0.038 mm/yr at 2 years and 0.028 mm/yr at 4 years in the lovastatin group compared with mean increases of 0.019 mm/yr at 2 years and 0.015 mm/yr at 4 years in the placebo group ($P < 0.001$). Reductions in IMT were greater in lovastatin recipients with higher baseline IMT values, whereas increases in placebo recipients were similar irrespective of baseline values. On-treatment levels of plasma LDL-C and several other plasma lipid measures correlated with the rate of change in IMT. Plasma LDL-C concentration decreased by 45.4\%–from 4.03 to 2.20 mmol/L (156 to 85 mg/dL)–in the lovastatin group, and remained unchanged in the placebo group.

A substudy of the Regression Growth Evaluation Statin Study (REGRESS)\textsuperscript{18} showed that pravastatin has a treatment effect on both the carotid and femoral artery walls. In this report from REGRESS, 255 men ($< 70$ years) with angiographic coronary disease and plasma total cholesterol levels of 155 to 310 mg/dL received pravastatin 40 mg daily or placebo and had carotid and femoral artery IMT measured at 6-month intervals for 2 years. The mean combined IMT decreased by 0.05 mm in the pravastatin group and remained unchanged in the placebo group, and the mean values for maximal IMT decreased by 0.005 mm and increased by 0.001 mm, respectively. The pravastatin treatment effects were highly significant (combined IMT, $P = 0.0085$; combined far-wall IMT, $P < 0.0001$; both vs. placebo). Plasma LDL-C concentration was reduced by 28.7\%–from 4.36 to 3.11 mmol/L (168 to 120 mg/dL)–in the pravastatin group and remained unchanged in the placebo group. Interestingly, a separate arm of the study that assessed pravastatin efficacy by means of coronary angiography failed to demonstrate improvement at the same level of statistical significance as did the ultrasonography arm of the study, despite the larger sample size in the angiography arm ($n = 885$).\textsuperscript{19} The difference in the 2 study arms highlights the benefit of B-mode ultrasonography as a research tool in statin interventional trials.
Atherosclerosis measured by B-mode ultrasonography

Figure 2  Carotid intima-media thickness (IMT) measurements in patients receiving lovastatin 80 mg or placebo daily in the Monitored Atherosclerosis Regression Study (MARS). (Reprinted with permission from *Ann Intern Med*.17)

In the Atorvastatin Versus Simvastatin on Atherosclerosis Progression (ASAP) trial, 325 patients (aged 30 to 70 years) with heterozygous familial hypercholesterolemia – which poses a high risk for accelerated and severe atherosclerotic disease – received daily atorvastatin 80 mg (aggressive lipid lowering) or simvastatin 40 mg (conventional lipid lowering) for 2 years.20 Patients either were previously untreated or had on-treatment plasma LDL-C levels >4.5 mmol/L (174 mg/dL). Carotid IMT was significantly reduced (– 0.031 mm) in the atorvastatin group and significantly increased (+0.036 mm) in the simvastatin group; the difference between groups was statistically significant (P =0.0001). Plasma levels of LDL-C were decreased by 51.5% with atorvastatin 80 mg treatment (from 8.00 to 3.88 mmol/L [309 to 150 mg/dL]) and by 42.3% with simvastatin 40 mg (from 8.33 to 4.81 mmol/L [322 to 186 mg/dL]). As in ACAPS,15 this trial combined common and internal carotid IMT as a primary outcome measure, so results are not easily comparable with other studies that did not include internal carotid IMT.6

This ASAP trial demonstrated the advantages of aggressive plasma lipid lowering over conventional treatment with respect to progression of atherosclerosis. Does it follow that lowering plasma LDL-C concentrations even below the level recommended by ATP III guidelines provides additional treatment advantages? Results of the Arterial Biology for the Investigation of the Treatment Effects of Reducing Cholesterol (ARBITER) study21 suggest that this may be the case. In this study, 161 patients (mean age, 60 years) who met ATP III criteria for plasma lipid-lowering therapy received atorvastatin 80 mg or pravastatin 40 mg daily for 1 year, with carotid IMT measured at 6 and 12 months. Plasma levels of LDL-C were reduced by 48.6% (from 148 to 76 mg/dL) with atorvastatin 80 mg and by 29.0% (from 155 to 110 mg/dL) with pravastatin 40 mg, whereas IMT was reduced by 0.034 mm in the atorvastatin group and increased by 0.025 mm in the pravastatin group at 12 months (P =0.03) (Figure 3).
Despite the difficulties in comparing IMT data across statin interventional trials, the weight of evidence indicates that a greater magnitude of plasma LDL-C lowering is associated with a greater beneficial impact in terms of atherosclerotic regression. These findings are clearly supported by, and lend support to, the ATP III report, which emphasizes the need for more aggressive treatment of patients at risk for CVD. In fact, the results of the ARBITER study suggest that plasma LDL-C lowering to below currently recommended target levels provides additional benefit in atherosclerotic regression.

A new trial of rosuvastatin – the Measuring Effects on Intima Media Thickness: an Evaluation of Rosuvastatin (METEOR) study – is now exploring whether asymptomatic patients who are not candidates for plasma lipid-lowering therapy on the basis of ATP III risk assessment, but who have relatively high IMT values, can also benefit from statin treatment. Rosuvastatin is highly efficacious in reducing plasma concentrations of LDL-C as well as in improving other plasma lipid variables in hypercholesterolemic patients: when compared with atorvastatin, simvastatin, and pravastatin, rosuvastatin produced significantly greater reductions in plasma LDL-C values across the dose ranges of the drugs.

Rosuvastatin 40 mg daily reduces plasma levels of LDL-C by 63% in patients with mild to moderate hypercholesterolemia. In METEOR, a target population of 840 patients is to be randomized in the proportion of 5:2 to receive either double blinded rosuvastatin 40 mg or placebo daily for 2 years, respectively, with carotid IMT measured at 6-month intervals. Eligible patients are men aged 45 to 70 years and women aged 55 to 70 years who meet the following criteria: plasma LDL-C level of 3.1 to 4.1 mmol/L (120 to 159 mg/dL) plus 10-year coronary heart
disease risk <10% or plasma LDL-C level ≤4.9 mmol/L (189 mg/dL) and no additional risk factors; plasma high-density lipoprotein cholesterol level >1.6 mmol/L (6.2 mg/dL); plasma triglyceride level ≤1.65 mmol/L (500 mg/dL); and maximal carotid IMT of 1.2 to 3.5 mm. Trial participants thus are not candidates for plasma lipid-lowering therapy on the basis of ATP III risk assessment, which allows placebo use in the trial, but they have relatively high carotid IMT values. Rosuvastatin treatment is expected to reduce plasma LDL-C concentrations to well below 100 mg/dL, the upper end of the target range in patients with the most aggressive plasma LDL-C goals, as did atorvastatin 80 mg daily in the ARBITER study.

The METEOR study should provide important information on the effects of aggressive lipid reduction on regression of increased IMT in patients considered to be at low risk of clinical disease. A positive outcome will provide support for the use of statin treatment in individuals who are at low risk on the basis of standard risk assessment while having evidence of advanced atherosclerotic disease determined by means of IMT measurements. B-mode ultrasonography has also been used in clinical trials of other plasma lipid-lowering agents, such as ezetimibe. Because this technique allows disease progression and intervention effects to be assessed in smaller patient populations and over relatively shorter periods than in studies using clinical end points, B-mode ultrasonography is expected to be incorporated into the phase 3 programs of novel agents currently in development, including cholesteryl ester transfer protein inhibitors, acyl coenzyme A:cholesterol acyltransferase inhibitors, ileal bile acid transport inhibitors, microsomal triglyceride transfer protein inhibitors, and dual peroxisome proliferator-activated receptor–α and –γ agonists. For the full benefit of B-mode ultrasonography to be realized, however, several issues remain to be addressed, including further standardization of the imaging protocols.
References


Part II

Low-density lipoprotein cholesterol
High frequency of ApoB gene Mutations Causing Familial Hypobetalipoproteinemia in patients of Dutch and Spanish descent

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Jorge Peter
Sergio Castillo
Miguel Pocovi
Rodrigo Alonso
John J.P. Kastelein
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Abstract

Background: Familial hypobetalipoproteinaemia (FHBL) is an autosomal co-dominant hereditary disorder of lipoprotein metabolism characterised by decreased low density lipoprotein (LDL) cholesterol and apolipoprotein B (APOB) plasma levels. High levels of plasma APOB and LDL cholesterol are strong predictors for risk of cardiovascular disease (CVD), while individuals with low APOB and LDL cholesterol levels are thought to have lower than average risk for CVD, and in fact, heterozygous FHBL patients appear to be asymptomatic.

Methods: Rather than identifying truncated APOB proteins in plasma fractions separated by gel electrophoresis, which will miss any mutations in proteins smaller than 30 kb, we analysed the APOB gene directly, using PCR.

Results: We identified nine different mutations, six of which are novel. Each mutation showed complete co-segregation with the FHBL phenotype in the families, and statistically significant differences between carriers and non-carriers were found for plasma total, LDL, and HDL cholesterol, triglycerides, and APOB levels, but not for APOA1 levels. All carriers of an APOB mutation were completely free from CVD.

Conclusions: Prolonged low levels of LDL cholesterol and elevated levels of HDL cholesterol may reduce the progression of atherosclerotic disease, but this has not been unequivocally shown that this is indeed the case in individuals with FHBL, and is the subject of a current study.
Introduction

Familial hypobetalipoproteinemia (FHBL) is an autosomal co-dominant hereditary disorder of lipoprotein metabolism characterized by decreased low-density lipoprotein (LDL)-cholesterol and apolipoprotein B (apoB) plasma levels. ApoB is a key structural component of triglyceride and cholesterol-rich lipoproteins such as chylomicrons, very-low-density lipoproteins (VLDL) and LDL, and therefore plays a pivotal role in cholesterol metabolism. High levels of plasma apoB and LDL-cholesterol are strong predictors for risk of cardiovascular disease (CVD), while individuals with low apoB and LDL-cholesterol levels are thought to have lower-than-average risk for CVD. In fact, heterozygous FHBL patients appear to be asymptomatic. However, the accompanying clinical phenotype is not well defined, since only a few kindreds with a definite molecular diagnosis have been investigated in detail. The few reported symptomatic FHBL subjects suffered from diarrhoea, neurological manifestations, fatty liver, retarded growth, weight loss and vitamin A and E deficiency. In FHBL kindreds assessed at the molecular level, low LDL-cholesterol and apoB levels are caused by mutations in the gene encoding apoB-100. To date, over 40 different molecular defects have been reported and most of these mutations prevent the translation of a full-length apoB protein. The frequency of apoB gene mutations causing truncated apoB and FHBL is considered rare and estimated to occur in 1.4% to 2.7% of individuals with persistent low levels of total and LDL-cholesterol.

Due to the size of the apoB gene (spanning 43 kb of which 14 kb is translated into the apoB-100 protein), the commonly used approach was to identify truncated forms of the apoB protein by sodium dodecyl sulphate-polyacrylamide gel electrophoresis of delipidated VLDL and LDL plasma fractions. This strategy will only detect point mutations and frame-shift mutations that lead to truncated apoB proteins that are larger than apoB-30. Truncated proteins smaller than apoB-30 are not incorporated in lipoproteins but are rapidly degraded or retained in the endoplasmic reticulum. Additionally, apoB truncation between B-30 and B-32 are only present in the density range of HDL. Therefore, analyzing only VLDL and LDL plasma fractions failed to identify certain apoB gene mutations. Furthermore, evidence exists that other loci might also contribute to a low cholesterol trait. Linkage to these putative loci was found in two genetic localizations, encompassing 3p21.1-22.13 and 13q.14 In order to identify the cause of the FHBL phenotype in our probands, we chose to analyze the complete apoB gene by direct sequencing, rather than to analyze the apoB proteins in different lipoprotein fractions.

Material and Methods

Study subjects were selected by analysis of cholesterol levels collected during the course of a number of studies addressing several forms of genetic dyslipidemia. Selection criterion was a LDL-cholesterol level below the 5th percentile for sex and age. Secondary causes for low
LDL-cholesterol levels, i.e. vegetarian, diet poor in fat or cancer were excluded. The probands were of Dutch or Spanish descent and provided information on their own health status and the structure of their kindreds. Blood samples were obtained from probands and their relatives after an overnight fast of at least 12 hours. All study subjects provided written informed consent and the study protocol was approved by the institute’s Ethical Review Board.

Plasma concentrations of total cholesterol, HDL-cholesterol and triglycerides were measured by commercially available kits (Boehringer Mannheim, Mannheim, Germany). LDL-cholesterol concentrations were calculated by the Friedewald formula only when the triglyceride concentration was below 4.5 mmol/L. ApoB and apoAI were determined on a Behring nephelometer BN100 using standard and references supplied by the manufacturer (Behring, Marburg, Germany).

Genomic DNA was prepared from 10 ml whole blood on an AutopureLS apparatus according to manufacturer’s protocol (Gentra Systems, Minneapolis, MI, USA). To analyse the promoter region, all 29 exons and the intronic boundaries of the ApoB gene, fifty-four pairs of primers were designed. PCR amplification was carried out with 50 ng of genomic DNA in a 25 µl reaction volume containing 1x Taq DNA Polymerase buffer (Qiagen, Hilden, Germany), 50 µM of each dNTP, 0.4 µM of each primer, 5 µg BSA and 1 unit Taq DNA polymerase. The thermal cycling conditions were as followed: 96°C for 5 min once, then 35 cycles of 96°C for 20 sec, 55°C to 60°C (depending on primer CG content) for 20 sec, and 74°C for 30 sec in a PCR apparatus (T3 Biocycler, Biometra, Germany). The sequence reactions were performed using fluorescently labelled dideoxy chain terminations with the Big Dye Terminator ABI Prism Kit (Applied Biosystems, Foster City, CA, USA) according manufactory’s protocol and analyzed on an Applied Biosystems Model 3730 automated DNA sequencer. Sequences were analyzed with the Sequencher package (GeneCodes Co, Ann Arbor, MI, USA).

All data were analysed using SPSS software (version 10.1, SPSS, Chicago, IL, USA) by ANOVA and by multiple linear regression analyses with adjustment for age and sex. A p-value of less than 0.05 was considered to be statistically significant.
Results

We identified 32 individuals meeting our inclusion criterion. After sequence analysis of the apoB gene, we identified 9 different mutations in 14 of our probands (Table 1).

<table>
<thead>
<tr>
<th>Exon</th>
<th>Mutation</th>
<th>WT</th>
<th>MT</th>
<th>Bp position</th>
<th>Predicted size</th>
<th>Family</th>
</tr>
</thead>
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<td>R412X&lt;sup&gt;17&lt;/sup&gt;</td>
<td>CGA</td>
<td>TGA</td>
<td>1315</td>
<td>ApoB-9</td>
<td>SP809</td>
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<tr>
<td>11</td>
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<td>CGG</td>
<td>TGG</td>
<td>1468</td>
<td>NA</td>
<td>SP810</td>
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<tr>
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<td>2534delA</td>
<td>A</td>
<td>delA</td>
<td>2534</td>
<td>ApoB-18</td>
<td>NL804</td>
</tr>
<tr>
<td>18</td>
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<td>delC</td>
<td>2783</td>
<td>ApoB-20</td>
<td>NL826, NL827</td>
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<td>TAA</td>
<td>4006</td>
<td>ApoB-29</td>
<td>NL808, NL822</td>
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<td>CCA</td>
<td>TGA</td>
<td>7600</td>
<td>ApoB-55</td>
<td>NL809, SP812</td>
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<td>11548delTT</td>
<td>TT</td>
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<td>11548</td>
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<td>NL825</td>
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<td>26</td>
<td>11712delC&lt;sup&gt;19&lt;/sup&gt;</td>
<td>C</td>
<td>delC</td>
<td>11712</td>
<td>ApoB-86</td>
<td>NL801, NL802, NL806</td>
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</tbody>
</table>

The reference sequence used is NM_000384, with the A of the ATG-translation initiation codon numbered nucleotide +1 and the methionine numbered as amino acid –27.

The R412X<sup>17</sup> and 11712delC<sup>19</sup> mutations, resulting in truncated apoB-9 and apoB-86 proteins, respectively, and the missense mutation, R463W<sup>18</sup>, were described previously. Additionally, we identified six novel apoB gene mutations resulting in truncated apoB protein of different sizes. The frame shift causal deletion of nucleotides AT at base pair 1718 (1718delAT) in exon 13 resulted in a stop codon at amino acid position 547, which leads to a predicted apoB-12 protein. Deletion of an adenosine at nucleotide 2534 (2534delA) in exon 17, causes a frame shift resulting in amino acid changes running from amino acid 818 to 834 and finally a stop codon at amino acid 835, leading to an apoB-18 protein. The deletion of a cystidine at base pair 2783 (2783delC), resulted in amino acid changes running from amino acids 902 to 924 and finally a stop codon at 925, which leads to a truncated apoB-20 protein. A nonsense mutation comprising a single C to T transition of nucleotide 4006 in exon 25, thereby creating a Ddel restriction site, changes the codon for glutamine at amino acid 1309 into a stop codon (Q1309X), leading to a predicted apoB-29 protein. The C to T substitution at position 6700 in exon 26, converting an arginine at amino acid 2507 into a stop codon (R2507X). The predicted protein contains 2506 amino acid residues, and is designated as apoB-55. Finally a deletion of TT at base pair position 11548 in exon 26 resulting in a stop codon at amino acid 3823 (11548delTT) leading to an predicted apoB-84 protein.

Screening for the frame-shift mutations was performed by direct sequencing of the relevant region of the apoB gene and both nonsense mutations were screened by PCR followed by digestion with the appropriate restriction enzyme. Since the R2507X mutation did not introduce or delete a restriction site, a mutagenic forward primer was designed that substituted an A at nucleotide position 7598 with a C, creating an NlaIII restriction site when the R2507X mutation

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was present. The six novel mutations found were screened in a group of 94 normolipemic controls, in which none of the mutations were found. Moreover, each mutation showed complete co-segregation with the FHBL phenotype in the families.

Clinical information on each FHBL family is listed in Table 2. Individuals with and without the FHBL trait did not differ significantly from each other with regard to BMI or apoA1 levels after adjustment for age and sex. Statistically significant differences between affected and unaffected groups were found for plasma total cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides and apoB levels (all p-values < 0.001). Within the group of patients with FHBL we could not establish a relation between LDL-cholesterol and apoB levels and the size of the truncated apoB protein.

Although most heterozygous FHBL patients appear to be asymptomatic, some individuals did have complaints that may be associated with low LDL-cholesterol and apoB levels. The proband of the NL806 family indicated that he was suffering from occasional episodes of diarrhoea. The proband of the NL808 family was a 59-year-old male, who was referred to our Lipid Clinic because of high glucose levels and was diagnosed with Diabetes Mellitus (DM) type 2. Medical examination revealed extremely low cholesterol levels and severe obesity with a BMI of 39.2 kg/m². Glucose levels remained high after medication. Medical examination of the 33-year-old male proband of the NL809 family, revealed DM type I at age 31 and neurological complaints of anaesthesia in his feet and paraesthesia in his hands. Vitamin A levels were slightly elevated (>3.9 µmol/l) and vitamin E levels were low (12 µmol/l). The diabetes was well managed by diet and insulin. Vitamin E levels returned to normal after oral administration of 400 mg vitamin E daily, after which his neurological complaints diminished.

Of the 27 individuals with persistent low levels of total and LDL-cholesterol and a proven hereditary trait in their families, 14 were identified with a functional apoB mutation, representing a disease frequency of 52%. In 18 probands we were not able to identify a causal apoB gene mutation to explain the low cholesterol levels. To demonstrate linkage or exclusion of linkage of the apoB gene to the low cholesterol phenotype we attempted to perform family investigation in all. However, not enough relatives were available for linkage analysis in eight of these kindreds. In five cases, family investigation showed no discernible pattern of the low cholesterol trait. In another five cases it was evident that the low cholesterol trait was present due to other causes than mutations in the apoB gene (Figure 1).
Table 2  Clinical characteristics of FHBL carriers and their unaffected relatives.

<table>
<thead>
<tr>
<th>family status</th>
<th>N</th>
<th>M/F</th>
<th>AGE</th>
<th>BMI</th>
<th>TC</th>
<th>LDL</th>
<th>HDL</th>
<th>TG</th>
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<th>APOA1</th>
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<td>56</td>
<td>31/25</td>
<td>39.7±18.1</td>
<td>25.5±4.7</td>
<td>5.23±1.22</td>
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<td>1.01±0.28</td>
<td>1.41±0.25</td>
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<tr>
<td>NL802 carriers</td>
<td>4</td>
<td>3/1</td>
<td>49.0±19.2</td>
<td>26.0±17.0</td>
<td>2.68±0.82</td>
<td>1.07±0.52</td>
<td>1.23±0.25</td>
<td>0.86±0.92</td>
<td>0.43±0.14</td>
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<td>NL804 carriers</td>
<td>35</td>
<td>18/17</td>
<td>34.9±21.6</td>
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<td>4.97±1.08</td>
<td>2.84±0.85</td>
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<td>NL808 carriers</td>
<td>4</td>
<td>3/0</td>
<td>52.3±5.9</td>
<td>39.2</td>
<td>2.80±0.75</td>
<td>1.03±0.31</td>
<td>1.61±0.70</td>
<td>0.38±0.40</td>
<td>0.29</td>
<td>1.00</td>
</tr>
<tr>
<td>NL809 carriers</td>
<td>3</td>
<td>3/0</td>
<td>47.5±24.7</td>
<td>nm</td>
<td>4.56±0.16</td>
<td>2.26±0.58</td>
<td>1.69±1.16</td>
<td>1.35±0.95</td>
<td>0.83±0.33</td>
<td>1.66±0.61</td>
</tr>
<tr>
<td>NL822 carriers</td>
<td>1</td>
<td>0/1</td>
<td>53.0</td>
<td>25.7</td>
<td>7.11</td>
<td>5.18</td>
<td>1.23</td>
<td>1.55</td>
<td>nm</td>
<td>nm</td>
</tr>
<tr>
<td>NL825 carriers</td>
<td>6</td>
<td>3/3</td>
<td>35.8±22.0</td>
<td>21.8±3.8</td>
<td>4.50±0.94</td>
<td>2.70±0.75</td>
<td>1.45±0.27</td>
<td>0.72±0.28</td>
<td>0.81±0.21</td>
<td>1.45±0.21</td>
</tr>
<tr>
<td>NL826 carriers</td>
<td>7</td>
<td>3/4</td>
<td>38.8±23.5</td>
<td>21.4±3.4</td>
<td>3.57±1.15</td>
<td>1.64±1.15</td>
<td>1.70±0.35</td>
<td>0.49±0.33</td>
<td>0.52±0.32</td>
<td>1.57±0.23</td>
</tr>
<tr>
<td>NL827 carriers</td>
<td>4</td>
<td>2/2</td>
<td>28.3±18.5</td>
<td>22.7±5.9</td>
<td>4.81±0.85</td>
<td>2.08±0.94</td>
<td>1.50±0.50</td>
<td>1.88±0.16</td>
<td>0.88±0.27</td>
<td>1.57±0.21</td>
</tr>
<tr>
<td>NL828 carriers</td>
<td>5</td>
<td>3/2</td>
<td>40.2±25.6</td>
<td>24.0±6.2</td>
<td>2.30±0.83</td>
<td>0.77±0.76</td>
<td>1.19±0.47</td>
<td>1.02±0.12</td>
<td>0.31±0.13</td>
<td>1.28±0.29</td>
</tr>
<tr>
<td>NL829 carriers</td>
<td>8</td>
<td>2/6</td>
<td>60.5±22.2</td>
<td>24.7±3.7</td>
<td>5.14±0.83</td>
<td>3.10±0.56</td>
<td>1.61±0.22</td>
<td>0.96±0.25</td>
<td>0.96±0.18</td>
<td>1.61±0.18</td>
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<tr>
<td>NL830 carriers</td>
<td>9</td>
<td>6/3</td>
<td>54.3±21.3</td>
<td>26.3±5.2</td>
<td>3.00±0.60</td>
<td>1.00±0.26</td>
<td>1.71±0.59</td>
<td>0.66±0.50</td>
<td>0.33±0.07</td>
<td>1.56±0.35</td>
</tr>
<tr>
<td>SP807 carriers</td>
<td>2</td>
<td>2/0</td>
<td>45.0±33.9</td>
<td>26.7±2.9</td>
<td>2.37±1.27</td>
<td>1.12±0.77</td>
<td>0.97±0.23</td>
<td>0.92±0.95</td>
<td>0.23±0.09</td>
<td>nm</td>
</tr>
<tr>
<td>SP809 carriers</td>
<td>1</td>
<td>0/1</td>
<td>12.0</td>
<td>19.5</td>
<td>4.74</td>
<td>3.21</td>
<td>0.95</td>
<td>1.23</td>
<td>0.93</td>
<td>0.97</td>
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<tr>
<td>SP810 carrier</td>
<td>1</td>
<td>1/0</td>
<td>15</td>
<td>23.5</td>
<td>1.71</td>
<td>0.49</td>
<td>1.06</td>
<td>0.49</td>
<td>0.14</td>
<td>nm</td>
</tr>
<tr>
<td>SP812 carrier</td>
<td>1</td>
<td>1/0</td>
<td>36.0</td>
<td>24.7</td>
<td>3.81</td>
<td>2.24</td>
<td>1.06</td>
<td>1.11</td>
<td>0.31</td>
<td>nm</td>
</tr>
<tr>
<td>carriers, htz</td>
<td>2</td>
<td>1/1</td>
<td>60.5±3.5</td>
<td>26.3±1.27</td>
<td>2.85±0.38</td>
<td>0.85±0.33</td>
<td>1.78±0.18</td>
<td>0.48±0.25</td>
<td>0.29±0.02</td>
<td>nm</td>
</tr>
<tr>
<td>carriers, hom</td>
<td>2</td>
<td>1/1</td>
<td>28.0±14.0</td>
<td>23.2±0.04</td>
<td>2.02±0.64</td>
<td>0.07±0.09</td>
<td>1.80±0.51</td>
<td>0.44±0.08</td>
<td>nd</td>
<td>nm</td>
</tr>
<tr>
<td>All carriers</td>
<td>133</td>
<td>63/70</td>
<td>40.0±20.3</td>
<td>24.2±5.2</td>
<td>5.14±1.18</td>
<td>3.15±1.02</td>
<td>1.46±0.47</td>
<td>1.18±0.92</td>
<td>0.95±0.29</td>
<td>1.49±0.29</td>
</tr>
<tr>
<td>All carriers, htz</td>
<td>88</td>
<td>51/32</td>
<td>41.3±20.0</td>
<td>24.8±4.9</td>
<td>2.80±0.66</td>
<td>0.95±0.46</td>
<td>1.67±0.47</td>
<td>0.43±0.49</td>
<td>0.32±0.14</td>
<td>1.52±0.26</td>
</tr>
</tbody>
</table>

Age is shown in years. Lipids (TC: total cholesterol, LDL: LDL-cholesterol, HDL: HDL-cholesterol and TG: triglyceride) are in mmol/l, Apolipoprotein B (ApoB) and apolipoprotein AI (ApoA1) are in mg/dl. nm: not measured; nd: not detectable; htz: heterozygous; hom: homozygous; M: male; F: female.
Figure 1  Composition of the proband cohort studied after DNA analysis of the apoB gene.

Discussion

In 14 out of 32 probands with low cholesterol levels we were able to identify an apoB gene mutation, resulting mainly in truncated forms of apoB. Although the functionality of these mutations was not validated in a strictest sense, it is well established that truncated apoB proteins are the cause of FHBL. Moreover, all mutations co-segregated with the FHBL phenotype, and therefore it seems likely that these variants are the cause of the FHBL phenotype in our families. Statistically significant differences between carriers and non-carriers were found for plasma total cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides and apoB levels, but not for apoA1 levels. The low levels of LDL-cholesterol are the result of failure to produce normal amounts of VLDL from truncated apoB proteins. This leads to a reduced conversion from VLDL-particles to LDL particles as normally occurs through the action of lipoprotein lipase. Additionally, a reduction of the activity of cholesterylster transfer protein (CETP) through low numbers of VLDL-particles results in a reduced transfer rate of cholesterolesters from HDL to VLDL and of triglycerides from VLDL to HDL, thus leading to elevated HDL-cholesterol and reduced triglyceride levels. The normal level of apoA1 in both carriers and non-carriers is in line with similar HDL-particle numbers in both groups, albeit with a different composition in term of cholesterolesters and triglycerides.

Some of the FHBL patients presented with a mild clinical phenotype not definitely linked to their lipoprotein disorder. In one case, occasional episodes of diarrhoea were noted, consistent with FHBL and in another case mild neurological symptoms were found, that diminished after supplementation with vitamin E. The two cases of diabetes were in all likelihood not related to FHBL. The combination of DM and FHBL, with or without neurological complaints, has been described before in 5 cases and our patients do not seem to differ clinically from patients examined by others.20-23
One interesting case was an 8-year old girl, initially diagnosed with Familial Defective Apolipoprotein B (FDB) caused by the R3500Q mutation that she inherited from her father. However, her lipid profile (TC 3.71 mmol/l; LDL-C 2.35 mmol/l; HDL-C 1.25 mmol/l; TG 0.23 mmol/l; apoB 0.54 g/l) did not match the phenotypic characteristic of FDB. The subsequent identification of the 11712delC mutation, inherited from her mother, explained her normal cholesterol level. Since the girl had no complaints associated with neither FDB nor FHBL, we could assume that the resulting phenotypic expression is a consequence of the compensation of one disorder by the other.

The precise prevalence of apoB gene mutations causing truncated apoB and FHBL is not known. However, several larger studies in individuals with persistent low levels of total and LDL-cholesterol show an estimated frequency between 1.4% and 2.7%. From these studies, it appears that truncated apoB is rare in healthy subjects with low LDL-cholesterol levels. However, these data are very different from the frequency of 52% for the mutations we identified in our study population. This discrepancy might be explained by a number of different factors. Firstly, we only included individuals free from any secondary causes of hypocholesterolemia. Secondly, we applied very strict in- and exclusion criteria for enrolment and lastly, our cohort was substantially larger than any other previously studied. However, additionally, this difference could be explained by the approach used, since we choose to analyze the apoB gene by direct sequencing, rather than to analyze the apoB protein. The assessment of lipoprotein fractions would have failed to detect most of our mutations, since they represent truncated apoB proteins smaller than apoB-30.

Interestingly, we were not able to identify a causal apoB gene mutation in all FHBL patients. Although, we did sequence at least 50 base pairs into each intron and analyzed up to 600 base pairs upstream of the promoter region, the presence of mutations outside these regions could not be ruled out, nor the presence of a large deletion or insertion. Additionally, yet unidentified genes could be the cause of the FHBL phenotype in these kindreds, since evidence is accumulating that other genetic factors besides the apoB gene may lead to a FHBL trait, such as loci identified on chromosome 3p21.1-22. and chromosome 13q. Identification of these putative genes would provide novel insights into the mechanisms operating in apoB metabolism.

It is well established that high levels of plasma apoB are strong predictors for risk of cardiovascular disease (CVD), but less is known about this risk in individuals with a FHBL phenotype. In our study, all carriers of an apoB mutation were completely free from cardiovascular disease. It can be hypothesized that prolonged low levels of LDL-cholesterol and elevated levels of HDL-cholesterol will reduce the progression of atherosclerotic disease. Nevertheless, it has not been unequivocally shown that this is indeed the case in individuals with FHBL. Assessment of the thickness of the intima-media complex (IMT) in individuals with FHBL, compared with non-affected siblings could be used to test this hypothesis. Such a study is currently under way in our centre and is the subject of a future report.
Acknowledgements

We gratefully acknowledge the contribution of the physicians K. Hovingh, E. Stroes, and W. van Dorp of the Dutch Lipid Clinics by submitting blood samples of FHBL patients. We also wish to thank our genetic field worker, Mrs. Holtkamp, for collection of blood samples of family members. This work was supported by a grant 2000B138 of the Netherlands Heart Foundation.
References


22. Groenewegen WA. Apolipoprotein B-38.9 does not associate with apo[a] and forms two distinct HDL density particle populations that are larger than HDL. *J Lipid Res* 1994;35:1012-25.

Hepatic and Cardiovascular Consequences of Familial Hypobetalipoproteinemia

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Sigrid W. Fouchier
Stefan de Haan
Barbara A. Hutten
Eric de Groot
John J.P. Kastelein
and Erik S.G. Stroes

Abstract

Objective: Individuals with familial hypobetalipoproteinemia (FHBL) have been reported to be prone to fatty liver disease (FLD). Conversely, the profound reduction of LDL-cholesterol in this disorder might decrease cardiovascular risk. In the present study, we assessed hepatic steatosis as well as non-invasive surrogate markers for cardiovascular disease (CVD) in subjects with FHBL and in matched controls.

Methods: Hepatic steatosis was assessed by abdominal ultrasonography. Carotid intima-media thickness (IMT) and distal common carotid arterial wall stiffness as surrogate markers for CVD risk were measured using high-resolution B-mode ultrasonography.

Results: Whereas transaminase levels were only modestly elevated, both prevalence (54% versus 29%; p=0.01) and severity of steatosis were significantly higher in FHBL individuals compared to controls. In spite of similar IMT measurements, arterial stiffness was significantly lower in FHBL (p=0.03) compared to controls. Additionally, the increase in arterial stiffness as seen in the presence of traditional risk factors was attenuated, suggesting that very low levels of apoB-containing lipoproteins can negate the adverse effects of other risk factors on the vasculature.

Conclusions: FHBL is characterized by an increased prevalence and severity of fatty liver disease. The observed decreased level of arterial wall stiffness, most pronounced in the presence of non-lipid risk factors, is indicative of cardiovascular protection in these subjects.
Introduction

Familial hypobetalipoproteinemia (FHBL) is a hereditary disorder of lipoprotein metabolism characterized by very low levels of apolipoprotein (apo) B-100. Plasma levels below the fifth percentile are distinctive for this condition which inherits as an autosomal dominant trait. The prevalence in the general population is estimated to vary from 0.1% to 1.9%. Genetic causes of hypobetalipoproteinemia include FHBL, abetalipoproteinemia and chylomicron remnant disease (OMIM numbers 601519, 246700 and 200100, respectively). A small percentage of FHBL can be explained by mutations in the gene encoding apolipoprotein B-100 (APOB). These include nonsense, frame-shift, and splicing mutations. Recently, it was reported that a missense mutation in the APOB gene can also lead to FHBL. APOB gene mutations lead to truncated forms of apoB and are characterized by slower hepatic secretion as well as more rapid plasma clearance compared to wild-type apoB-100 particles. Since apoB is the main constituent of such lipoproteins, including very low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL) and low-density lipoprotein (LDL), FHBL subjects are characterized by exceptionally low levels of these pro-atherogenic particles from birth onwards. Whereas subjects with heterozygous FHBL are generally asymptomatic, two potential implications have been attributed to this particular condition. First, the impairment of hepatic VLDL-TG secretion in FHBL may contribute to fat accumulation in the liver. Potential consequences of fat accumulation are highlighted by the occurrence of hepatic steatosis in subjects with non-alcoholic steatohepatitis (NASH), in whom progression towards cirrhosis has been observed. Indeed, case reports and smaller studies have reported a relationship between fatty liver disease (FLD) and FHBL. This is best illustrated by two recent studies by Schonfeld et al who showed that hepatic fat percentage, as assessed by magnetic resonance spectroscopy (MRS), was significantly increased in subjects with FHBL as compared to healthy controls. Nevertheless, the natural course of this potential fat accumulation in FHBL is as yet unknown. Second, FHBL offers a unique opportunity to evaluate the impact of life-long exposure to unusually low levels of apoB-containing, atherogenic lipoproteins. In this respect, FHBL subjects might be regarded as a natural model for ‘intensive lipid-lowering therapy’. In fact, the potential success of intensive lipid-lowering therapy has recently been reinforced by data from the REVERSAL and PROVE-IT study, showing a more pronounced reduction of cardiovascular disease risk upon intensive lowering of LDL-C levels.

In the present study we evaluated the impact of FHBL on hepatic steatosis as well as on surrogate markers for cardiovascular disease. Here we present the results of these investigations.
Methods

Subjects and protocols
For a detailed description of methods please visit http://atvb.ahajournals.org.
Eighty-two subjects were enrolled on study, 41 with FHBL and 41 healthy controls, matched for sex and body mass index (table 1). Autosomal codominant inheritance was a necessary characteristic for the clinical diagnosis of FHBL. FHBL subjects were identified from a group of individuals who were referred to our Lipid Clinic because of extreme low LDL-levels. These subjects were characterized by direct sequencing of the entire apoB gene, as published previously. Secondary causes for low LDL-C levels, i.e. (strict) vegetarian diet, or generalized diseases such as cancer, were excluded. The controls consisted of unaffected family members as well as unrelated volunteers. In the FHBL group, 4 subjects had diabetes mellitus (DM) compared to none in the control group. Since DM is strongly associated with both liver steatosis and carotid IMT/arterial stiffness we repeated the analyses after excluding the 4 diabetic subjects in the FHBL-group (FHBL minus DM) to assess whether possible differences between groups were obscured by the presence of DM.

Liver ultrasound
In all subjects ultrasound examination of the liver was performed by a single radiologist, blinded to the disease state of the subjects. The extent of hepatic fatty infiltration was classified according to previously published criteria.

Carotid Ultrasound
B-mode ultrasound intima-media thickness (IMT) measurements were performed in the far walls of the carotid arteries and M-mode arterial stiffness was measured bilaterally in the common carotid arteries.

Statistical analysis
Statistical analyses were performed using linear or logistic regression analyses with generalized estimating equations in the SAS procedure GENMOD to account for correlations within families. Analyses were performed using SAS software (release 8.02 SAS Institute Inc, Cary, NC, USA). A p-value < 0.05 was considered significant.
Table 1  Clinical characteristics for FHBL subjects and controls

<table>
<thead>
<tr>
<th></th>
<th>FHBL</th>
<th>Controls</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=41</td>
<td>n=37 (minus DM)</td>
<td>n=82*</td>
</tr>
<tr>
<td>Age (y)</td>
<td>41.1 ± 16.9</td>
<td>39.58 ± 16.54</td>
<td>45.8 ± 16.0</td>
</tr>
<tr>
<td>Male sex (%)</td>
<td>27 (66)</td>
<td>24 (65)</td>
<td>27 (66)</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>25.7 ± 5.1</td>
<td>25.0 ± 16.3</td>
<td>24.9 ± 3.7</td>
</tr>
<tr>
<td>Waist to hip ratio</td>
<td>0.86 ± 0.10</td>
<td>0.85 ± 0.10</td>
<td>0.87 ± 0.09</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>67 ± 12</td>
<td>67 ± 13</td>
<td>70 ± 12</td>
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<tr>
<td>Pulse pressure (mmHg)</td>
<td>57 ± 13</td>
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</tr>
<tr>
<td>SBP (mmHg)</td>
<td>138 ± 19</td>
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<td>136 ± 25</td>
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<tr>
<td>DBP (mmHg)</td>
<td>81 ± 12</td>
<td>80 ± 12</td>
<td>80 ± 11</td>
</tr>
<tr>
<td>Hypertension‡ (%)</td>
<td>3 (7)</td>
<td>1 (3)</td>
<td>3 (7)</td>
</tr>
<tr>
<td>DM (%)</td>
<td>4 (10)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Alcohol (U/day)</td>
<td>0.5 (0.0-1.3)</td>
<td>0.5 (0.0-1.8)</td>
<td>1.0 (0.0-2.0)</td>
</tr>
<tr>
<td>Smokers (%)</td>
<td>21 (51)</td>
<td>19 (51)</td>
<td>13 (33)</td>
</tr>
<tr>
<td>Pack-years of smokers</td>
<td>21.0 (10.5-30.8)</td>
<td>18 (10-28.5)</td>
<td>10.0 (7.3-19.5)</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD or number (percentage), except for alcohol consumption and pack-years which are given as median (interquartile range). * P-value indicates difference between FHBL group (n=41) and controls (n=41). † P-value indicates difference between FHBL-DM group (n=37) and controls (n=41). FHBL indicates familial hypobetalipoproteinemia; DM, diabetes mellitus; SBP, systolic blood pressure; DBP, diastolic blood pressure. ‡ Hypertension: SBP > 140 mmHg and/or DBP > 90 mmHg. § A p-value could not be calculated.

Results

Clinical characteristics of FHBL subjects and controls are presented in table 1. Forty-one subjects who met the clinical criteria of FHBL (apoB and LDL-C < fifth percentile adjusted by age, gender and race) participated in the study. Thirty-three of these subjects had mutations in the apoB gene, characteristic of FHBL. These genetically affected subjects were recruited from 8 families with different apoB mutations. The following apoB mutations were identified in the FHBL group: 2534delA apoB-18, Q1309X apoB-29, R2507X apoB-55, 11712delC apo-B8617 (Table 2). Subjects did not use lipid-lowering drugs. There was no significant difference in blood pressure, smoking, body mass index or alcohol consumption between FHBL subjects and controls. In line with their diagnosis, apoB-, LDL-C and total cholesterol levels were significantly lower in the FHBL group. The type of apoB truncation, but not plasma LDL-C or apoB levels, was modestly correlated with the degree of liver steatosis using Spearman’s rho method (r = 0.336, p=0.002). Mean levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and gamma-glutamyltransferase (GGT) levels were significantly higher in the FHBL group as compared to the controls (Table 3).
Table 2  Apolipoprotein B mutations

<table>
<thead>
<tr>
<th>exon</th>
<th>mutation</th>
<th>WT</th>
<th>MT</th>
<th>Bp position</th>
<th>predicted size</th>
<th># of carriers</th>
</tr>
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<tr>
<td>17</td>
<td>2534delA</td>
<td>A</td>
<td>delA</td>
<td>2534</td>
<td>ApoB-18</td>
<td>14</td>
</tr>
<tr>
<td>25</td>
<td>Q1309X</td>
<td>CAA</td>
<td>TAA</td>
<td>4006</td>
<td>ApoB-29</td>
<td>4</td>
</tr>
<tr>
<td>26</td>
<td>R2507X</td>
<td>CGA</td>
<td>TGA</td>
<td>7600</td>
<td>ApoB-55</td>
<td>1</td>
</tr>
<tr>
<td>26</td>
<td>11712delC</td>
<td>C</td>
<td>delC</td>
<td>11712</td>
<td>ApoB-86</td>
<td>14</td>
</tr>
</tbody>
</table>

The reference sequence used was NM_000384, with the A of the ATG translation initiation codon numbered nucleotide +1 and the methionine numbered as amino acid -27. (Adapted from Fouchier et al. J Med Genet. 2005 Apr;42(4):e23)

Table 3  Laboratory characteristics for FHBL subjects and controls

<table>
<thead>
<tr>
<th></th>
<th>FHBL</th>
<th>Controls</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=41</td>
<td>n=37 (minus DM)</td>
<td>n=82*</td>
</tr>
<tr>
<td>TC (mmol/L)</td>
<td>2.95 ± 0.88</td>
<td>2.96 ± 0.88</td>
<td>5.26 ± 0.95</td>
</tr>
<tr>
<td>HDL-C (mmol/L)</td>
<td>1.73 ± 0.59</td>
<td>1.76 ± 0.60</td>
<td>1.55 ± 0.33</td>
</tr>
<tr>
<td>LDL-C (mmol/L)</td>
<td>1.04 ± 0.50</td>
<td>1.02 ± 0.50</td>
<td>3.07 ± 0.76</td>
</tr>
<tr>
<td>TG (mmol/L)</td>
<td>0.40 (0.18-0.55)</td>
<td>0.39 (0.17-0.53)</td>
<td>1.06 (0.77-1.81)</td>
</tr>
<tr>
<td>ApoB (g/L)</td>
<td>0.36 ± 0.13</td>
<td>0.35 ± 0.12</td>
<td>0.92 ± 0.22</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>5.0 (4.8-5.1)</td>
<td>4.9 (4.8-5.1)</td>
<td>4.9 (4.6-5.2)</td>
</tr>
<tr>
<td>hs-CRP (mg/L)</td>
<td>1.7 (0.8-3.0)</td>
<td>1.8 (1.0-3.0)</td>
<td>1.6 (0.7-3.5)</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>30 (23-35)</td>
<td>30 (23-35)</td>
<td>25 (22-30)</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>32 (22-54)</td>
<td>32 (22-54)</td>
<td>23 (17-32)</td>
</tr>
<tr>
<td>GGT (U/L)</td>
<td>25 (18-43)</td>
<td>25 (18-43)</td>
<td>19 (15-35)</td>
</tr>
<tr>
<td>Alk. Phos. (U/L)</td>
<td>62 (51-73)</td>
<td>62 (53-73)</td>
<td>67 (54-80)</td>
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</tbody>
</table>

Data are presented as mean ± SD, except for glucose, TC, TG, hs-CRP, ALT, AST, GGT and Alk. Phos. which are given as median (interquartile range). * P-value indicates difference between FHBL group (n=41) and controls (n=41). † P-value indicates difference between FHBL-DM group (n=37) and controls (n=41). FHBL indicates familial hypobetalipoproteinemia; DM, diabetes mellitus; TC, total cholesterol; HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol; TG, triglycerides; ApoB, apolipoprotein B; hs-CRP, high-sensitivity C-reactive protein; Alk. Phos., alkaline phosphatase.

However, this was mainly caused by the 7 subjects with severe steatosis who had the highest levels of transaminases. Three of these 7 subjects had ALT levels more than twice the upper limit of normal (ULN) but still < 3x ULN. The highest value of ALT, observed in a diabetic patient, was 102. None of these 7 subjects had AST values > 2x ULN. AST and ALT were both significantly associated with hepatic steatosis (p=0.04 and p= 0.0002, respectively). Levels of hs-CRP and glucose were comparable between the two groups. HDL-C levels were significantly higher in the FHBL minus DM group compared to the controls (1.76 ± 0.59 mmol/L vs. 1.55 ± 0.33 mmol/L, p=0.01).

Liver Ultrasound

We observed a significantly higher prevalence of liver steatosis in the FHBL group compared to the control group (54% vs. 29%; p=0.01). In addition, FHBL subjects were also characterized by a more severe degree of hepatic steatosis. Seven (17%) of the subjects in the FHBL group were classified as severe steatosis compared to none in the control group (Figure 1). The distribution...
of steatosis severity was significantly different between groups (p=0.004). This was even more apparent when comparing the controls with the subgroup of FHBL with mutations (p=0.001). The 4 diabetic subjects were equally distributed over the 4 steatosis categories. Separate analyses with exclusion of these 4 subjects lead to an equal statistical difference for steatosis severity between groups.

Figure 1. Grade of Liver Steatosis

Hepatic steatosis was not more severe in those subjects carrying truncated apoBs not secreted into the plasma (apoB-18 and ApoB-29) compared to the carriers of longer truncations (p=0.68). Plasma LDL-C and apoB levels were also not different between these subgroups (p=0.77 and p=0.81, respectively). Nine of the 14 carriers of Apo-86 had liver steatosis compared to 3 of the 8 FHBL subjects without mutations. FHBL was positively associated with liver steatosis on univariate analysis (p=0.02). When adjusted for gender and smoking on multivariate analysis, FHBL, age, and BMI were independent predictors for the development of liver steatosis (p=0.001, p<0.0001 and p=0.0014, respectively). Similar significant results were found when running the analyses with the subgroup of FHBL subjects with mutations (n=33) compared to the healthy controls. Results were not significant when this was done for the subgroup of FHBL without mutations (n=8). However, the latter is likely to be caused by a lack of power due to the limited number of subjects in this group.

Vascular measurements

Mean carotid IMT (± SD) was 0.63 ± 0.14 mm in the FHBL group compared to 0.65 ± 0.15 mm in the control group. The latter difference on univariate analysis (p=0.049) lost significance when adjusting for age, gender, smoking and BMI on multivariate analysis. Arterial stiffness was significantly lower in the FHBL minus DM group compared to the controls on univariate analysis (p=0.04) whereas a similar trend was seen for the whole FHBL group (p=0.06). When comparing
the 2 subgroups of FHBL, (with and without mutations), FHBL was still significantly associated with arterial stiffness in the first group (p=0.04) but this was not significant for the subgroup without mutations. Again, this could reflect a lack of power, since the subgroup without mutations contained only 8 subjects. To evaluate a potential interaction between risk factors and apoB-containing lipoproteins, we attributed a cumulative risk score to each subject. The cumulative risk score comprised age, systolic blood pressure and smoking. These variables were chosen because their individual relationship with cardiovascular risk has been proven beyond any doubt as attested to by their incorporation in both the PROCAM\textsuperscript{27} and Framingham Risk Score\textsuperscript{28}, the 2 most widely used risk calculators for predicting cardiovascular disease. Moreover, these risk factors have an independent, strong association with arterial stiffness.\textsuperscript{29-35} In our study these parameters were also strongly correlated with arterial stiffness with the exception of smoking (r = 0.732, p<0.001, r = 0.550, p<0.001 and r = 0.267, p=0.018, respectively). In line, both FHBL and control subjects showed a gradual increase in arterial stiffness with increasing cumulative risk scores. However, using linear regression analysis, the slope for the FHBL group was markedly decreased compared to the controls, indicating decreased stiffening in the presence of non-lipid risk factors in the FHBL group (p=0.03) (Figure 2). This difference remained significant (p=0.04) when comparing the FHBL minus DM group with the control subjects.

Figure 2. Arterial Stiffness versus Cumulative Risk Score in FHBL

The Cumulative Risk Score is based on 3 variables: age, smoking and systolic blood pressure (SBP). Results of each variable, except for smoking, were divided into tertiles. For age and SBP, patients received scores of 1, 2 or 3 with each increasing tertile. Smoking was scored as either 0 for non-smoking or 3 for smoking. Minimal and maximal attainable scores were 2 and 9, respectively.

The p-value indicates the difference in slope between the two regression lines.
Discussion

Subjects with FHBL are characterized by an increased prevalence of fat accumulation in the liver as well as by a more severe degree of such hepatic steatosis. Whereas these findings are not novel, we show that FHBL subjects exhibit decreased arterial stiffness. Notably, the increase in arterial stiffness under the influence of ‘traditional’ non-lipid risk factors was markedly attenuated in FHBL subjects.

Biochemical analyses

Subjects with FHBL are characterized by significantly decreased levels of apoB-containing lipoproteins, including low LDL-C as well as low triglyceride-rich particles. Also, slightly higher levels of HDL-C were found in these FHBL subjects. Presumably, the latter is the consequence of low levels of triglycerides, thus minimizing exchange of cholesterol esters from HDL-C to apoB-containing particles through the action of cholesterol ester transfer protein. Another explanation could be that the truncated apoBs are only present in the density range of HDL. However, we excluded the latter option by performing agarose gel electrophoresis on HDL-fractions, from patients with apoB-55 and apoB-86, which were isolated after ultracentrifugation. No LDL-bands were present in the HDL-density range. Additionally, apoB could also not be detected in the HDL fraction using immunonephelometry (data not shown).

Hepatosteatosis

The prevalence of fatty liver disease in healthy controls (29%) is in the same order of magnitude as reported by others.9,19 The increased prevalence of hepatosteatosis in subjects with FHBL is in agreement with previous results from Schonfeld and Tanoli who showed that these subjects had a ~3-fold increase in mean liver fat content, as assessed by MRS.13,14 The most likely cause for this increase in hepatic steatosis is the impaired secretion of VLDL-TG from the liver, leading to accumulation of VLDL-TG in the liver. There is a large body of evidence suggesting that accumulation of liver triglycerides may give rise to increased oxidative stress in the hepatocytes.37-39 However, distinct proof, in the human setting, that this process invariably translates into progression of liver steatosis to NASH is lacking.40 Recent work by Youssef and colleagues revealed that up to 25% of patients with FLD may progresses to NASH, of whom 20% may eventually even progress into cirrhosis.19-20 Notably, in our FHBL group, transaminase levels were only modestly elevated with none of the subjects exceeding a three-fold increase in ULN. Most studies reporting long term outcome of fatty liver disease, use the AST/ALT ratio as a marker for the risk of disease progression. A ratio of less than 1 indicates a ‘low risk’ for steatosis41,42 and in our FHBL subjects, AST/ALT ratios were all below 1. It should be kept in mind, however, that the absence of liver enzyme elevations8,43 does not completely preclude advanced fibrosis or cirrhosis in these subjects.43 To date, long-term follow-up data with regard to liver outcome in FHBL are lacking. Nevertheless, risk factors for FLD such as hypertriglyceridemia, obesity, alco-
hol, diabetes mellitus and certain drugs are likely to aggravate hepatic steatosis in FHBL. Hence, it is prudent to avoid these risk factors, and recommend a diet with low to moderate amounts of fat and energy, limited use of alcohol as well as avoiding obesity in these individuals.

**Cardiovascular Risk**

Numerous studies have established a strong correlation between levels of LDL-C and progression of IMT. In the present study, however, we could not show an independent statistical difference in terms of carotid IMT values between FHBL subjects and controls. Nevertheless, data have accumulated recently, that show the predictive value of the assessment of vascular function, such as arterial stiffness, for future cardiovascular events. Arterial stiffness is closely correlated with increasing age, smoking and hypertension. The impact of these risk factors is augmented in the presence of hypercholesterolemia and can be reverted by statin therapy.

In our FHBL group, we observed a significant decrease in arterial stiffness. Of note, this difference was observed in spite of the fact that traditional risk factors such as smoking and diabetes occurred more frequently in the FHBL group compared to controls. In earlier studies, apoB-containing lipoproteins have been put forward as a pivotal factor for the development of atherogenic changes of the vessel wall. To evaluate a potential interaction between apoB-containing lipoproteins and other traditional risk factors, we constructed a cumulative risk index including age, smoking, and systolic blood pressure in FHBL subjects as well as controls. In both groups, there was a linear relationship between increased risk score and arterial stiffness. Interestingly, the increase in arterial stiffness, also in presence of these risk factors was decreased significantly in the FHBL group compared to controls. These data suggest that apoB-containing lipoproteins indeed have the ability to potentiate the impact of traditional risk factors on vascular function. Tentatively, these observations might suggest that lowering of apoB-containing lipoproteins should have a beneficial impact also in subjects with ‘non-cholesterol’ risk factors. Indeed, recent studies have validated the beneficial effects of statin therapy in normocholesterolemic subjects with non-lipid risk factors, such as hypertension.

This study has some limitations. We used the less sensitive ultrasonography method to evaluate fatty liver disease rather than magnetic resonance spectroscopy. However, in view of the carefully standardized methodology and the fact that both patients and controls were evaluated using the same methodology, it is unlikely that the latter has affected our outcomes. With regard to the IMT measurement, we could not find a clear relationship between LDL-C levels and carotid IMT. Several reasons may have attributed to the absence of a relation. First, we studied a relatively young cohort with an inherently low risk for cardiovascular disease and hence low IMT values. Second, we studied IMT in a case control design to show thinner IMTs compared to healthy controls. A priori, it is very difficult to demonstrate decreased IMT thickness in ‘low-risk’ groups compared to healthy controls. We have estimated that inclusion of more than 1000 subjects per group would have been necessary to be able to detect signifi-
cantly thinner IMTs compared to healthy controls with a “normal” risk factor distribution, as seen in western populations.

In summary, our study shows that subjects with FHBL are at increased risk of developing FLD. Whereas long-term sequelae of FLD in FHBL subjects remain to be established, it is prudent to give lifestyle advice in affected individuals. As is illustrated by decreased vascular wall stiffness, our findings suggest that the vessel wall in FHBL subjects is relatively protected by the (life-long) reduced levels of exposure to apoB-containing lipoproteins. The attenuated gradual increase in vascular stiffness in the presence of classical, non-lipid cardiovascular risk factors in FHBL subjects is of interest and suggests that apoB-containing particles constitute a central factor in atherogenesis, amplifying any risk mediated by non-lipid risk factors. Further confirmation of this finding is needed in larger cohorts to ascertain its impact on cardiovascular risk.

**Acknowledgements**

The cooperation of all study subjects is greatly appreciated. We are grateful to Patrick Rol and Johan Gort for assistance with carotid ultrasound examinations and to Dr. Nico Smits for assistance with liver data analysis. Sigrid W. Fouchier is supported by the Netherlands Heart Foundation (grant 2000B138). John J.P. Kastelein is an established investigator of the Netherlands Heart Foundation (2000D039).
References


Ezetimibe/Simvastatin (Inegy™) in the Treatment of Hyperlipidemia

J.J.P. Kastelein and R.R. Sankatsing

Summary

Ezetimibe/simvastatin (Inegy™), a dual inhibitor of both cholesterol production and absorption, is a new approach to the management of hyperlipidemia. Recent studies have shown that it produces greater reductions in low-density lipoprotein (LDL) cholesterol than the single inhibition of statin therapy, enabling many more patients to achieve their LDL cholesterol treatment goals. With ezetimibe/simvastatin therapy, reductions of up to 61% from baseline have been seen in LDL cholesterol, with clear improvements in other associated lipid fractions. It has been well tolerated across all studies, with a safety profile similar to that of statin therapy. This paper will review clinical experience to date with ezetimibe/simvastatin, commenting upon its place and potential value in the prevention of cardiovascular disease.
Introduction

Despite the clear risks of hyperlipidemia and the proven benefits of lipid lowering therapies, only a minority of patients currently achieve recommended low-density lipoprotein (LDL) cholesterol treatment goals in clinical practice (1-5). More patients are being treated for lipid reduction than ever before, but there still remains a substantial degree of under treatment. Although this may be due to a number of reasons (e.g. patient noncompliance, tolerability issues, variable physician follow-up), the most likely explanation is that patients are not receiving adequate dosages of the lipid lowering drugs available, or that the drugs themselves are not optimal. Either way, a more aggressive approach to LDL cholesterol reduction is warranted.

Until recently, clinicians had only been able to inhibit one source of cholesterol with drug therapy, that of cholesterol production. Ezetimibe/simvastatin (Inegy™), provides Dual Inhibition of both cholesterol production and absorption, representing a a new approach to lipid management. Recent large-scale, randomized, controlled clinical trials have shown that ezetimibe/simvastatin produces substantially greater reductions in LDL cholesterol than statin therapy, while maintaining a similar safety and tolerability profile to statin therapy (6-11). As such, it may be a viable alternative to traditional statin therapy.

This paper will review the data to consider the use of ezetimibe/simvastatin in the treatment of hyperlipidemia.

Ezetimibe/simvastatin: components with complementary actions

Simvastatin is a competitive inhibitor of hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase, the last regulated step in the synthesis of cholesterol. By lowering serum LDL cholesterol levels (through a combination of LDL receptor up-regulation and reduced entry of LDL cholesterol into the circulation), statins as a class have been shown to reduce the incidence of coronary artery disease by 25-60% and the risk of death from any cause by an approximate 30% (12). Simvastatin was one of the first statins to be associated with substantial improvements in morbidity and mortality in this respect (13), and has since shown benefit across a wide range of at-risk individuals (14).

Ezetimibe is the first in a new class of cholesterol absorption inhibitors that blocks the intestinal absorption of dietary and biliary cholesterol, without affecting the uptake of triglycerides or fat soluble vitamins (15, 16). As would be expected from its mode of action, ezetimibe has demonstrated significant reductions in LDL cholesterol in patients with primary hypercholesterolemia (p < 0.01 versus placebo), with favorable effects on associated lipid variables such as triglycerides and high-density lipoprotein (HDL) cholesterol (17, 18).
As blood cholesterol levels are maintained through both endogenous synthesis and intestinal absorption, an agent that inhibits both sources of cholesterol would be expected to lower LDL cholesterol levels to a greater extent than one that acts through either mechanism alone (Figure 1). This theory has proven correct in the laboratory and in the clinic. In a hypercholesterolemic dog model, for example, ezetimibe was seen to synergistically reduce plasma cholesterol levels in the presence of HMG-CoA reductase inhibitors (19). Early clinical pharmacology studies also demonstrated that simvastatin and ezetimibe had incremental benefit on LDL cholesterol reduction, and without any adverse drug-drug interactions (20). Subsequent clinical studies have since shown repeatedly that ezetimibe/simvastatin provides reductions in LDL cholesterol over and above those achieved with statin therapy. Key data in this respect are detailed below.

**Clinical efficacy of ezetimibe/simvastatin**

**Reductions in LDL Cholesterol**

Recently published findings from a 12-week treatment study enrolling 887 patients with primary hypercholesterolemia (LDL cholesterol 145-250 mg/dl; triglycerides ≤350 mg/dl) showed ezetimibe/simvastatin to be significantly (p < 0.001) more effective than simvastatin alone in reducing LDL cholesterol levels (8). In the study, patients were randomized to one of four different treatment regimens: ezetimibe 10 mg; simvastatin 10, 20, 40 or 80 mg; ezetimibe 10 mg plus simvastatin 10, 20, 40 or 80 mg; or placebo. Pooled data across all ezetimibe/simvastatin patients demonstrated a mean 53.2% reduction from baseline in LDL cholesterol compared with a 38.5% reduction for simvastatin alone (p < 0.001). The figure of 53.2% is important, as evidence suggests that a reduction in LDL cholesterol of at least 50% is needed for plaque stabilization and the reduced progression of coronary atherosclerosis (21). The differential between the treatment groups could be seen at each dose comparison. Reductions in LDL cholesterol of up to 61% from baseline were reported in patients treated with ezetimibe/simvastatin 10/80 mg (Figure 2). Maximal lowering of LDL cholesterol was evident by 2 weeks, and efficacy maintained throughout the study. In addition to the beneficial effects on LDL cholesterol, there were clear improvements in a number of other lipid fractions and inflammatory markers, with significance between the treatment groups for the majority of variables (Table 1). Of note, the data presented in this review relates to the co-administration of ezetimibe and simvastatin and not the co-formulation of the drugs. However, a recent study by Bays et al clearly demonstrated the bioequivalence of the ezetimibe/simvastatin (eze/simva) combination tablet to co-administration of the 2 individual drugs (22). In this study administration of eze/simva 10/10 mg, 10/20 mg, 10/40 mg and 10/80 mg reduced mean LDL-C levels with 44.8%, 51.9%, 55.2% and 60.2% respectively, after 12 weeks of treatment.
Figure 1  Dual inhibition of cholesterol production and absorption with ezetimibe/simvastatin

LDL Cholesterol Goal Attainment

Of important clinical consequence is that ezetimibe/simvastatin has been shown to allow more patients to reach their LDL cholesterol goal at a lower dose of simvastatin and with fewer dose titrations than simvastatin alone. In a 23-week study of 710 randomized, high-risk patients (men and women with LDL cholesterol ≥ 130 mg/dl meeting NCEP Adult Treatment Panel III criteria for coronary heart disease [CHD] or CHD risk equivalent), ezetimibe/simvastatin 10/10, 10/20 or 10/40 mg produced greater reductions in LDL cholesterol and allowed more patients to reach an LDL cholesterol treatment goal of < 100 mg/dl than simvastatin monotherapy (20 mg) (6) (Figure 3). After 5 weeks of treatment, 75% patients treated with ezetimibe/simvastatin 10/10 mg achieved LDL-C levels < 100 mg/dl compared to only 46% of patients treated with simvastatin 20 mg. Essentially, patients treated with ezetimibe/simvastatin10/10 mg had approximately 3.6 times greater odds of reaching their treatment goal than patients treated with simvastatin 20 mg. The corresponding odds for patients in the ezetimibe/simvastatin 10/20 and 10/40 mg groups were 6.0 and 8.4 times, respectively. In addition, relatively few patients in the ezetimibe/simvastatin groups required up-titration of the simvastatin dose. For example, 75% of patients receiving ezetimibe/simvastatin 10/10 mg reached their LDL cholesterol goal without a simvastatin dose titration, compared with fewer than half of patients receiving simvastatin 20 mg.
Figure 2  Percent reduction in LDL cholesterol levels from baseline to study endpoint: ezetimibe/simvastatin vs simvastatin monotherapy (8)

*<p < 0.001 vs corresponding simvastatin monotherapy dose

Table 1  Mean percent change from baseline to study end point (last available LDL cholesterol measurement) in lipid variables and inflammatory markers: ezetimibe/simvastatin vs simvastatin monotherapy (8)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Pooled simvastatin data</th>
<th>Pooled ezetimibe/simvastatin data</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>Mean % change from baseline (SD)</td>
</tr>
<tr>
<td>LDL cholesterol</td>
<td>345</td>
<td>-38.5 (14.2)</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>345</td>
<td>-26.4 (11.3)</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>345</td>
<td>7.6 (11.9)</td>
</tr>
<tr>
<td>Non-HDL cholesterol</td>
<td>345</td>
<td>-34.1 (13.8)</td>
</tr>
<tr>
<td>Triglycerides (median)</td>
<td>345</td>
<td>-15.2 (34.1)</td>
</tr>
<tr>
<td>Apo B</td>
<td>328</td>
<td>-29.2 (14.3)</td>
</tr>
<tr>
<td>Apo A-I</td>
<td>328</td>
<td>6.3 (13.8)</td>
</tr>
<tr>
<td>Apo A-II</td>
<td>199</td>
<td>2.8 (11.5)</td>
</tr>
<tr>
<td>Apo E</td>
<td>328</td>
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</tr>
<tr>
<td>Lp(a)</td>
<td>199</td>
<td>-9.6 (39.3)</td>
</tr>
<tr>
<td>LDL: HDL cholesterol</td>
<td>345</td>
<td>-42.0 (15.2)</td>
</tr>
<tr>
<td>Total cholesterol: HDL cholesterol</td>
<td>345</td>
<td>-30.8 (13.0)</td>
</tr>
<tr>
<td>CRP (median)</td>
<td>204</td>
<td>-8.7 (61.7)</td>
</tr>
<tr>
<td>Fibrinogen (median)</td>
<td>198</td>
<td>4.4 (20.0)</td>
</tr>
</tbody>
</table>

*p < 0.001 versus pooled simvastatin; LDL = low-density lipoprotein; HDL = high-density lipoprotein; Apo = apolipoprotein; Lp = lipoprotein; CRP = C-reactive protein
Other Patient Populations

Preliminary data also suggest a broader clinical role for ezetimibe/simvastatin for patients where greater LDL cholesterol reduction is necessary, the compound demonstrating notable efficacy in groups of patients at high risk of cardiovascular disease. In patients with type II diabetes mellitus, for example, ezetimibe/simvastatin 10/20 mg was more effective in reducing LDL cholesterol than doubling the dose of statin therapy, and enabled the majority of patients to meet their LDL cholesterol treatment goals (23, 24). It also improved levels of C-reactive protein, a sensitive marker for cardiovascular risk in these patients (25).

Safety and tolerability of ezetimibe/simvastatin

Ezetimibe/simvastatin has been evaluated for safety in more than 3200 patients in clinical trials. Studies reported to date have shown ezetimibe/simvastatin to be well tolerated, with a safety profile similar to that of statin monotherapy. No clinically meaningful differences have been seen between ezetimibe/simvastatin and either simvastatin or atorvastatin as single agents in terms of overall adverse events (drug-related or not), or clinical/laboratory adverse events leading to discontinuation of treatment (6-8) (Table 2). Importantly, there were no reported cases of rhabdomyolysis in clinical trials. Nevertheless, vigilance is required as there have been some case reports of patients experiencing myopathy/tendinopathy both with and without increased serum creatine kinase activity after adding ezetimibe to a statin (26).
Table 2  Summary of adverse events across three randomized, controlled studies comparing ezetimibe/simvastatin with statin monotherapy (6-8)

<table>
<thead>
<tr>
<th></th>
<th>Simvastatin</th>
<th>Atorvastatin</th>
<th>Ezetimibe/simvastatin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20 mg</td>
<td>Pooled†</td>
<td>10 mg</td>
</tr>
<tr>
<td>Any clinical adverse events</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Study 1</td>
<td>168 (66%)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Study 2</td>
<td>-</td>
<td>-</td>
<td>187 (71%)</td>
</tr>
<tr>
<td>Study 3</td>
<td>-</td>
<td>219 (63%)</td>
<td>-</td>
</tr>
<tr>
<td>Treatment-related adverse events</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Study 1</td>
<td>19 (8%)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Study 2</td>
<td>-</td>
<td>-</td>
<td>42 (16%)</td>
</tr>
<tr>
<td>Study 3</td>
<td>-</td>
<td>46 (13%)</td>
<td>-</td>
</tr>
<tr>
<td>Discontinuation due to any adverse events</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Study 1</td>
<td>14 (6%)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Study 2</td>
<td>-</td>
<td>-</td>
<td>10 (4%)</td>
</tr>
<tr>
<td>Study 3</td>
<td>-</td>
<td>7 (2%)</td>
<td>-</td>
</tr>
<tr>
<td>Creatine kinase ≥10 times ULN</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Study 1</td>
<td>2 (1%)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Study 2</td>
<td>-</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Study 3</td>
<td>-</td>
<td>1 (0.3%)</td>
<td>-</td>
</tr>
<tr>
<td>Alanine aminotransferase and/or aspartate aminotransferase ≥3 times ULN</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Study 1</td>
<td>0</td>
<td>-</td>
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<tr>
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<tr>
<td>Study 3</td>
<td>-</td>
<td>0</td>
<td>-</td>
</tr>
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</table>

† Simvastatin 10, 20, 40 or 80 mg; ULN = upper limit of normal; study 1 = Feldman et al 2004 (6); study 2 = Ballantyne et al 2004 (7); study 3 = Goldberg et al 2004 (8)

Efficacy comparisons with other statins

**Ezetimibe/Simvastatin vs Atorvastatin**

In addition to comparisons of ezetimibe/simvastatin versus simvastatin alone, the published literature also provides evidence for the superior efficacy of ezetimibe/simvastatin versus atorvastatin in the treatment of hypercholesterolemia. A recently reported forced-titration study compared the efficacy of ezetimibe/simvastatin with atorvastatin in 788 patients randomized to i) atorvastatin (10 mg titrated to 20, 40 and 80 mg at 6-week intervals); ii) ezetimibe/simvastatin (10/10 mg titrated to 10/20, 10/40 and 10/80 mg at 6-week intervals); and iii) ezetimibe/simvastatin (10/20 mg titrated to 10/40 mg after 6 weeks and 10/80 mg after 18 weeks) (7). All patients were 18 years or older, with baseline LDL cholesterol levels at or above the drug treatment threshold detailed in the National Cholesterol Education Program (NCEP) Adult Treatment Panel III guidelines (27). After the first 6 weeks of treatment (primary endpoint), ezetimibe/simvastatin (10/10 and 10/20 mg) produced significantly greater reductions in LDL cholesterol (-46% and -50%, respectively) than atorvastatin 10 mg (-37%; p ≤ 0.05). In fact, at all time/dose points throughout the study, ezetimibe/simvastatin showed greater efficacy than
atorvastatin in decreasing LDL cholesterol (Figure 4), as well as non-HDL cholesterol, apolipoprotein B, and total cholesterol. Similarly, ezetimibe/simvastatin was significantly ($p \leq 0.05$) more effective than atorvastatin in increasing levels of HDL cholesterol from baseline.

**Ezetimibe in Combination with Atorvastatin, Pravastatin, Lovastatin and Rosuvastatin**

The beneficial effects of ezetimibe coadministered with a statin are not limited to simvastatin; valuable improvements in clinical efficacy have also been seen in combination with all statins studied, such as atorvastatin (9, 28-30), as well as pravastatin (31), lovastatin (32), and rosuvastatin (33). For example, in a recent pooled analysis of data from a collective 2382 patients with primary hypercholesterolemia, 12 weeks of treatment with ezetimibe plus one of four statins (atorvastatin, lovastatin, pravastatin or simvastatin) produced significantly ($p < 0.01$) greater reductions in LDL cholesterol, total cholesterol, triglycerides, non-HDL cholesterol and apolipoprotein B compared with statin therapy alone (34). HDL cholesterol levels were also significantly ($p < 0.01$) increased. At each statin dose, coadministration with ezetimibe led to a greater LDL cholesterol reduction than the next highest statin monotherapy dose. Moreover, the enhanced LDL cholesterol lowering effects of ezetimibe plus statin were independent of the statin type, and were generally consistent across patient subgroups (e.g. age, gender, hypertension, diabetes, baseline lipid level, and family history of CHD). The safety profiles of all ezetimibe/statin combinations were similar to each other and to those of statin therapy alone.

**Figure 4** Percent reduction in LDL cholesterol levels from baseline with ascending doses of ezetimibe/simvastatin vs atorvastatin monotherapy (7)
Considerations

There is a wide variation in the response to ezetimibe with some cases reported of patients with hypercholesterolemia who are unresponsive at all to treatment with ezetimibe. It has been suggested that variants in the \textit{NPC1L1} gene, the molecular target for ezetemibe, are responsible for this unresponsive phenotype \cite{35}. Also, the additional benefit of adding ezetimibe to a statin in patients with refractory familial hyperlipidemia or patients who are intolerant to statin therapy is modest with an average 11\% additional reduction in LDL-C as recently reported by Wierzbicki et al \cite{36}. Incidences of ezetimibe-induced hyperlipidemia, both in monotherapy and in combination with statins have also been observed. Apart from biological variation and lesser dietary or drug compliance this could be explained by an ezetimibe-induced increase in hepatic cholesterol synthesis, albeit unlikely.

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<thead>
<tr>
<th>Study</th>
<th>Objective(s)</th>
<th>Measures</th>
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<tr>
<td>Ezetimibe and Simvastatin in Hypercholesterolemia Enhances Atherosclerosis Regression (ENHANCE)</td>
<td>To evaluate the effects of aggressive lipid lowering on carotid artery intima media thickness: ezetimibe/simvastatin vs statin monotherapy</td>
<td>Primary: Mean change in carotid artery intima media thickness Secondary: Incidence of plaque regression, changes in maximal intima media thickness</td>
<td>725 patients at 18 international centers treated for 2 years</td>
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<tr>
<td>Improved Reduction of Outcomes: VYTORIN™ Efficacy International Trial (IMPROVE IT)</td>
<td>To evaluate the risk reduction provided by ezetimibe/simvastatin vs simvastatin in reducing death and major coronary events in patients with acute coronary syndromes</td>
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<td>10,000 patients followed for at least 2 years</td>
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<tr>
<td>Simvastatin and Ezetimibe in Aortic Stenosis (SEAS) Study</td>
<td>To assess whether aggressive cholesterol lowering in patients with moderate AS slows progression of AS, reduces number of valve replacements, and incidence of CVD outcomes: ezetimibe/simvastatin vs placebo</td>
<td>Primary: Risk reduction in composite endpoint of MCEs$^1$ Secondary: Aortic valve events, echocardiographic progression of AS, safety/tolerability</td>
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<td>Study of Heart and Renal Protection (SHARP)</td>
<td>To assess the effects of ezetimibe/simvastatin vs placebo in patients with chronic kidney disease</td>
<td>Primary: Time to 1st major vascular event (nonfatal MI or cardiac death, nonfatal/fatal stroke, revascularization) Secondary: Progression to ESRD, various causes of death, MCEs$^2$, stroke, hospitalization for angina</td>
<td>9000 patients at &gt;200 hospitals in 10 countries treated for \geq 4 years</td>
</tr>
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\textit{MI} = myocardial infarction; \textit{ESRD} = end-stage renal disease; \textit{AS} = aortic stenosis; \textit{CVD} = cardiovascular disease; VYTORIN = INEGY

$^1$MCEs = cardiovascular death, aortic valve replacement surgery, CHF as a result of progression of AS, nonfatal MI, CABG, PCI, hospitalized unstable angina, nonhemorrhagic stroke; $^2$MCEs (major cardiovascular events) = nonfatal MI or cardiac death
In recent years, lipid lowering therapy has been directed towards the inhibition of cholesterol production through the use of statins. For many patients, however, clinical efficacy can only be achieved through a strategy of dual inhibition of both the production and absorption of cholesterol. As such, ezetimibe/simvastatin would appear to present the clinician and patient with a number of advantages over existing therapy. First, the impressive efficacy seen with ezetimibe/statin therapy should offer patients an increased likelihood of LDL cholesterol goal attainment. As large proportions of patients with hyperlipidemia currently remain under treated, an intervention that increases the chances of goal realization has to be viewed as positive. In fact, some might contend that even patients who comfortably achieve LDL cholesterol goals on existing therapy might benefit from more aggressive lipid lowering. There is much to support the ‘lower is better’ argument. Every major primary prevention trial of statin therapy to date has demonstrated that lower LDL cholesterol levels are associated with a reduced risk of atherosclerotic disease (37). Those that have analyzed event rates in relation to LDL cholesterol have shown that lower LDL cholesterol tertiles are associated with a reduced occurrence of major coronary events (38), and that aggressive lipid lowering can produce more favorable outcomes than conservative approaches (39-42). This has led some to propose that target LDL cholesterol levels should be as low as < 70 mg/dl, and not 100-115 mg/dl as recommended by current guidelines (43). As a matter of fact, the recently revised NCEP guidelines have already moved towards this new LDL-C target of < 70 mg/dl in (very) high-risk patients (44). A similar pattern can be noticed in the Joint British Societies guidelines II/ British Hypertension Society guidelines IV (45). These changes were brought about by the beneficial results of intensive lipid-lowering therapy beyond current targets observed in the PROVE-IT and Heart Protection Study (14, 41). From a clinical practice standpoint, multiple (upward) dose adjustments with ezetimibe/simvastatin therapy should not be necessary, and many more patients should be able to achieve their LDL cholesterol goals with low doses. In contrast, initial doses of statins are very often insufficient to enable patients to achieve their goals. Clinical evidence shows that when initial doses of statins are doubled, this only provides an additional 6% reduction in LDL cholesterol (12).

Future studies will need to address the potential clinical benefits of ezetimibe/statin therapy over and above those of improving LDL cholesterol levels. The body of evidence for simvastatin is clear in this respect, there being a strong patient outcomes base in the form of the Scandinavian Simvastatin Survival Study and Medical Research Council/British Heart Foundation Heart Protection Study (13, 14, 46). As a single agent, ezetimibe has been shown to reduce atherosclerotic progression in an animal model (47), but clinical evidence of the effectiveness of ezetimibe/simvastatin in the prevention of the complications of atherosclerosis is not yet available. An active outcomes program for ezetimibe/simvastatin is ongoing (Table 3) (48-50). These studies, which include over 21,000 patients across a number of countries worldwide,
will confirm whether the greater LDL cholesterol lowering effects of Dual Inhibition translate in the clinic into beneficial modifications of cardiovascular endpoints. Clearly, it would be an advancement in clinical practice to offer appropriate patients with hyperlipidemia the greater effectiveness of dual cholesterol inhibition with ezetimibe/simvastatin.

**Acknowledgements**

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References


Part III

HIV and Lipids
Atherosclerotic vascular disease in HIV: It is not just ART that Hurts the Heart!

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Abstract

**Purpose of review:** Although potent combination antiretroviral therapy (cART) has heralded an unparalleled improvement in the treatment of HIV-1 infected patients, the now well-known metabolic complications of treatment, which include dyslipidemia, insulin resistance and changes in body fat distribution are thought to contribute to an increased risk of atherosclerotic (cardio)vascular disease (CVD). Atherogenic changes in plasma lipids as well as some evidence of increased atherogenesis, however, had already been described in HIV-1 infected patients prior to the availability of cART and even prior to that of suboptimal antiretroviral therapy. In this review we will summarize the various possible factors and mechanisms involved in atherogenesis in HIV-1 infected individuals, with a focus on those mechanisms related to the infection itself and its immunological consequences.

**Recent findings:** Recent data suggest that a treatment strategy involving repeated cycles of CD4+ cell-guided cART interruption is associated with a higher risk of CVD than continuous treatment aimed at optimal viral suppression.

**Summary:** Apart from the effects of cART-associated metabolic derangements, HIV-1 infection directly or indirectly, for instance by being associated with a state of chronic immune activation, may contribute to atherogenesis.
1. Introduction

Although potent combination antiretroviral therapy (cART) has heralded an unparalleled improvement in the treatment of HIV-1 infected patients, the now well-known metabolic complications of treatment, which include dyslipidemia, insulin resistance and changes in body fat distribution are thought to contribute to an increased risk of atherosclerotic (cardio)vascular disease (CVD). Atherogenic changes in plasma lipids as well as some evidence of increased atherogenesis, however, had already been described in HIV-1 infected patients prior to the availability of cART.

2. HIV-1 infection and atherosclerosis

Almost ten years ago, it was first suggested that use of HIV protease inhibitors is associated with premature coronary artery disease. Subsequently, several studies, of which the D:A:D (Data Collection on Adverse Events of Anti-HIV Drugs) study is the largest prospective observational study, have been performed to address this question. The primary analysis of the D:A:D study, which involved over 23,000 HIV-1-infected patients, patients, indeed reported a 26% relative increase in the rate of myocardial infarction (MI) per year of exposure to cART, which was independent of the risk exerted by classical CVD risk factors. A more recent updated analysis from the same study involving a longer follow-up of more patients (almost 100,000 person years of follow-up), demonstrated that the previously reported association between cART exposure and CVD could indeed be attributed to the exposure to HIV protease inhibitors. This association was not found for exposure to non-nucleoside reverse transcriptase inhibitors (NNRTI). Part, but not all of the risk of protease inhibitor exposure could be explained by dyslipidemia associated with exposure to PI-containing cART regimens.

Apart from the potentially atherogenic effect of cART and HIV protease inhibitors in particular there is evidence, some of it quite recent, which suggests that HIV-1 infection either by itself or through its effects on the immune system may also contribute to acceleration of the atherosclerotic process. The initial indication for a possible role of HIV-1 infection in accelerating atherosclerosis came from studies dating back to long before the era of potent cART or even suboptimal dual combination therapy for that matter. As far back as the early 1990’s, autopsy studies reported extensive atherosclerotic lesions in the coronary arteries of young HIV-seropositive adults with a mean age of 27 and 32 years, respectively, and similar findings were reported even earlier in paediatric patients before the introduction of zidovudine. More specifically, Tabib et al. reported findings from a small series of eight autopsies demonstrating significant coronary lesions in young (23-32 year old) HIV-infected patients. None of these patients were known to have a positive family history for coronary heart disease or other known CVD risk factors. All 8 subjects showed fibrosis of their coronary arteries with foam cells, which
was widely disseminated and causing 40–50% occlusion of the coronary arterial lumen similar to what can be found in older individuals from the general population.

More recently, results from the SMART (Strategies for Management of Antiretroviral Therapy) trial, in which continuous antiretroviral therapy (the viral suppression arm or VS) was compared with episodic use of antiretroviral therapy guided by the peripheral blood CD4+ T cell count (the drug conservation arm or DC) have provided novel additional evidence that uncontrolled HIV-1 infection may contribute to the pathogenesis of coronary artery disease. Although the average proportion of time spent on antiretroviral therapy during follow-up was almost three times longer in the viral suppression than in the drug conservation arm (93.7% vs 33.4%, respectively) simply by virtue of the two treatment strategies being compared in SMART, the rate of fatal or nonfatal cardiovascular disease was found to be statistically significantly higher in the drug conservation group (hazard ratio 1.6 (95% CI 1.0–2.5) for DC versus VS arm). The concept that uncontrolled HIV replication and the cellular immunodeficiency and chronic immune activation associated with it may contribute to cardiovascular risk is also supported by a study using ultrasonographic measurement of the carotid artery intima-media thickness (cIMT), which is a validated and accepted marker for generalized atherosclerosis and vascular disease risk. In this study, mean IMT was significantly increased in 148 HIV-1-infected adults when compared to 63 HIV-1-negative age and sex-matched control subjects at study entry, and also progressed more rapidly over a one year follow-up period in those who were infected with HIV-1. In a multivariate analysis, both classic CVD risk factors as well as being HIV-1-infected were found to be independent predictors of increased cIMT at baseline, and a nadir CD4+ T cell count of ≤ 200 cells/mm³ was independently associated with cIMT progression.

In this review, the various ART-and non-ART associated factors and mechanisms thought to be involved in atherogenesis in HIV-1-infected individuals will be summarized, with a focus on those mechanisms related to the infection itself and its immunological consequences.

### 3. Metabolic complications of cART

The introduction of cART has heralded an unparalleled improvement in the treatment of HIV-1-infected patients, with AIDS-associated mortality rates being reduced to less than a fifth of that in the pre-cART era in a sustained manner. These therapies however have been found to be associated with now well known metabolic complications, which include dyslipidemia, insulin resistance and changes in body fat distribution each of which are thought to potentially contribute to an increased risk of atherosclerotic (cardio)vascular disease (CVD).

**Insulin Resistance**

Insulin resistance and type 2 diabetes mellitus are well-known risk factors for atherosclerotic vascular disease in the general population. In previously treatment-naive HIV-1-infected indi-
individuals, evidence of insulin resistance was observed in 13 percent during the first year of cART.\textsuperscript{14} HIV-1 infected patients with cART-associated lipodystrophy not only exhibit reduced uptake of glucose by skeletal muscle,\textsuperscript{15} but also have been found to be insulin resistant at the level of the liver and adipose tissue.\textsuperscript{16} Although both lipoatrophy and intraabdominal lipohypertrophy associated with cART likely contribute to reduced insulin sensitivity, it has been shown that a minor degree of reduction in insulin sensitivity may already occur early on following the initiation of protease inhibitor-containing cART and prior to demonstrable alterations in body fat distribution.\textsuperscript{17} These early changes may be related to the inhibitory effect of PI’s on the activity of the cellular glucose transporter 4 (GLUT-4).\textsuperscript{18} Interestingly, a recent study conducted in healthy uninfected volunteers has provided evidence that the NRTI stavudine may also directly induce insulin resistance, which was shown to be associated with reduced mitochondrial function in skeletal muscle.\textsuperscript{19} Thus, a variety of factors and mechanisms are likely to contribute to the pathogenesis of insulin resistance which may be observed in HIV-1-infected patients treated with cART.\textsuperscript{20}

**Dyslipidemia**

In addition to insulin resistance, cART has been suggested to enhance atherogenesis by inducing dyslipidemia, characterized predominantly by the elevation of circulating triglyceride-rich lipoproteins such as Very Low Density Lipoproteins (VLDL) and chylomicrons, that occurs shortly after the initiation of treatment.\textsuperscript{21} A recent meta-analysis showed that after 48 weeks of treatment with protease inhibitors, the proportional elevation in concentrations of total cholesterol, triglycerides, and low density lipoprotein cholesterol (LDL) were 66%, 80%, and 37%, respectively.\textsuperscript{22} In the D:A:D study triglyceride levels >200 mg/dL were present in 40% of PI-treated patients, 32% of those treated with NNRTIs, 23% of NRTI-treated patients, and 15% of the untreated patients.\textsuperscript{23} High density lipoprotein cholesterol (HDL) < 0.9 mmol/L was found in 27%, 19%, 25% and 26%, and increased total cholesterol >6.2 mmol/L in 27%, 23%, 10% and 8% in PI-, NNRTI-, NRTI-treated and untreated patients respectively. These data show that a PI-based antiretroviral regimen generally is associated with a more atherogenous lipid profile when compared to NNRTI-based regimens.

Apart from PI’s and NNRTI’s, use of the thymidine analogue NRTI’s, stavudine and to a lesser extent zidovudine may also affect lipid profiles. In a study of approximately 600 ART naive patients who were randomized to tenofovir DF or stavudine (combined with the NNRTI efavirenz and the NRTI lamivudine in all patients) the use of stavudine was associated with significant higher increases in fasting triglycerides (+1.51 vs +0.01 mmol/L), total cholesterol (+1.50 vs +0.78 mmol/L), LDL (+0.67 vs 0.36 mmol/L) and a significantly lower increase of HDL (+0.16 vs. +0.23 mmol/L).\textsuperscript{24} In another study, including over 500 ART naive patients randomized to either zidovudine plus lamivudine or tenofovir DF plus emtricitabine, plus efavirenz in both arms,\textsuperscript{25} patients randomized to zidovudine/lamivudine had a significantly higher increase in fasting total cholesterol (0.91 vs 0.54 mmol/L), and LDL (0.52 vs 0.34 mmol/L). Somewhat surprisingly,
HDL levels showed a significantly greater increase in the zidovudine-treated patients: 0.23 vs. 0.16 mmol/L). The difference in increase of triglycerides was not statistically different between the two groups. Stavudine is known to be more toxic in terms of mitochondrial toxicity than zidovudine, and tenofovir has not been demonstrated to exhibit mitochondrial toxicity. The effects of thymidine analogue NRTI on the lipid profile may be the result of direct toxic effects of these drugs on mitochondria and inhibition of beta-oxidation of fatty acids, and indirectly may also be affected by the association between the use of these drugs and the occurrence of both lipoatrophy and insulin resistance.

In terms of mechanisms, it has been suggested that elevation of circulating VLDL early in the course of cART is caused by the combination of impaired VLDL clearance, already present in untreated HIV-1 infected patients, as well as by a cART-mediated increase in VLDL secretion by the liver.

Similar to what is the case for the pathogenesis of insulin resistance, the pathophysiological mechanisms behind dyslipidemia induced by the different components of cART remain to be fully elucidated. This is complicated by the fact that HIV-1 infection by itself also affects levels of plasma lipids. In the early nineties it was already shown that triglycerides and free fatty acids were increased in patients with untreated HIV-1 infection, while plasma levels of HDL and LDL apolipoprotein-AI (apoAI) and apoB levels were decreased. These findings were confirmed in a cohort study of 50 documented HIV-1 seroconverters, which showed a decrease in total, HDL and LDL levels after seroconversion when compared to pre-infection values. After initiation of cART in these patients HDL levels hardly changed, but total and LDL levels increased. These increases therefore, at least partially, might reflect a restoration to pre-infection levels.

### 4. Immune activation

The observation that the atherosclerotic process is accelerated in patients infected with HIV-1 was made well before cART became available. As mentioned before, severe atherosclerotic lesion were found in the coronary arteries of chronically infected HIV-1-patients which could not be explained by classical risk factors. This is a strong indication that other mechanisms, in addition to the abovementioned metabolic complications of HIV treatment, are involved. In addition, HIV-1 infection was identified as an independent predictor of atherosclerosis progression in a study measuring cIMT in HIV-1 infected patients treated with cART. The authors suggested that the chronic immune activation associated with HIV-1 may explain these findings. In a recently published cross-sectional study comparing 93 HIV-infected and 37 uninfected adults, these same authors indeed presented evidence that the HIV-infected patients had higher CD4 and CD8-T cell activation and higher cytomegalovirus (CMV)-specific interferon-γ CD8 T-cell responses. Of note, the latter was independently associated with IMT, in the sense that for every 10-fold increase in the precentage of CMV-specific CD8 T-cells there was a 14 per-
cent increase in carotid IMT.\textsuperscript{32} The contribution of chronic low-grade immune activation and the chronic inflammation associated with it to the pathogenesis of atherosclerosis is well recognized.\textsuperscript{33} HIV-1 infection is indeed strongly associated with chronically increased immune activation and more pronounced in patients with more advanced cellular immunodeficiency.\textsuperscript{34,35} It is characterized by the presence of chronically activated T-cells, B-cells and monocytes/macrophages, as well as by increased expression of various leukocyte activation markers, production of pro-inflammatory cytokines and a rise in cell proliferation.\textsuperscript{36} Although the degree to which the immune system is activated may diminish with cART, it is not completely reversed even after years of sustained cART-induced viral suppression.\textsuperscript{37} Interestingly, it was recently suggested that depletion of gastrointestinal CD4\textsuperscript{+} T cells, which occurs very early after HIV-1 infection, compromises the integrity of the intestinal mucosal barrier and leads to increased translocation of bacteria from the intestinal lumen.\textsuperscript{38} As such bacteria and bacterial components subsequently stimulate innate immune cells systemically, creating the proinflammatory milieu associated with chronic HIV-1 infection.\textsuperscript{39} As a result of subsequent leukocyte activation, cytokine production and increased biomarkers HIV-1 infection may exert distinct pro-atherogenic effects on the vasculature of HIV-1 infected patients, similar to what can be seen in other systemic inflammatory conditions. Indeed, enhanced atherogenesis has also been observed in patients with systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and Crohn's disease (CD).\textsuperscript{40-43} As such, systemic inflammation has emerged as an universal risk factor for atherothrombotic disease.\textsuperscript{44,45} Several mechanisms secondary to systemic inflammation have been proposed which may explain the increased occurrence of atherothrombotic disease in patients with a condition associated with chronic immune activation and inflammation such as chronic HIV-1 infection.

**Lipoproteins**
A systemic inflammatory state induces dyslipidemia characterized by increased levels of triglycerides and decreased levels of HDL.\textsuperscript{46} Although initiation of cART has also been shown to induce dyslipidemia, this is preceded by dyslipidemic changes associated with HIV-1 infection itself, as we have outlined above. This notion is corroborated by various studies reporting dyslipidemia in HIV-1 infected patients in the pre-cART era\textsuperscript{30,47-49} and more recently by the Multicenter AIDS Cohort Study.\textsuperscript{31} In this study, serum samples of 50 male HIV-1 seroconverters were available from the following time points: preseroconversion (sample from the last seronegative visit), after seroconversion but before cART initiation and at several time points after cART initiation. Interestingly, HIV-1 infection itself resulted in notable declines in mean serum TC (–30 mg/dL [-0.78 mmol/L]), HDL (–12 mg/dL [-0.31 mmol/L]), and LDL values (–22 mg/dL [-0.57 mmol/L]). Unfortunately, changes in levels of triglycerides, insulin, and glucose were not quantified. Although initiation of cART resulted in increases in mean TC and LDL values, these levels, observed after years of cART, at least partially represented a return to preinfection serum lipid levels after accounting for expected age-related changes.\textsuperscript{31}
Although it has been well established that HDL plays a pivotal role in atheroprotection, this is of particular relevance in HIV-1 infected patients with acute coronary syndromes (ACS), since these were reported to have significantly lower HDL levels compared with HIV-uninfected ACS patients.\textsuperscript{50} HDL is well known as a negative acute phase protein and systemic inflammation can have a major negative effect on HDL levels. In the pre-cART era, decreased plasma concentrations of HDL in HIV-1 infected individuals were shown to be associated with immune activation.\textsuperscript{51} In line with this, in the D:A:D study, the risk of having decreased HDL was highest among patients with low CD4\textsuperscript{+} T cell count and high plasma HIV-1 RNA viral load.\textsuperscript{23} Indeed, HDL correlates negatively with current and peak viral load and positively with current and nadir absolute and percent CD4\textsuperscript{+} T cell count and CD4 percentage. Thus, considering HDL levels are reduced proportionally to the severity of HIV-1 infection, it appears this plays a major role in reducing HDL levels.\textsuperscript{52} It has been suggested that the effect of HIV-1 infection on HDL levels is greater than the cART effect.\textsuperscript{53} A relation between immune activation and dyslipidemia was also demonstrated in other studies.\textsuperscript{30,54} Taken together this implies that HIV-1 infection and HIV-1 associated chronic immune activation both have a significant atherogenic impact on lipid profiles. Of note, the non-nucleoside reverse transcriptase inhibitor nevirapine plays an interesting role as it may directly oppose infection- and immune activation-mediated reductions of HDL. A randomized trial in treatment-naive patients showed that a nevirapine-based cART regimen led to a prominent increase of both HDL (49\%) and apoAI (19\%) which was significantly greater than the changes in these levels seen in patients randomized to either an indinavir or lamivudine-based cART regimen, which in all arms was combined with the NRTI combination of stavudine and didanosine.\textsuperscript{55} These differences remained, after adjusting for changes in HIV-1 plasma RNA and CD4\textsuperscript{+} T-cell levels, indicating an effect of nevirapine on HDL and apoAI over and above that which may be explained by suppression of HIV-1 infection.\textsuperscript{56} It is still unknown whether this is a specific nevirapine effect or whether this might be a class-specific effect, since cART including the NNRTI efavirenz also increases HDL levels, albeit to a lesser extent than nevirapine-based treatment.\textsuperscript{56} The potential mechanisms underlying these changes remain speculative but unravelling thereof could contribute to understanding the lipoprotein disturbances in HIV-1 infected patients.

In addition to lowering HDL levels, a systemic inflammatory state may also affect the biochemical composition and thereby function of HDL. HDL is composed of various enzymes and other proteins which contribute significantly to its atheroprotective capacity such as the anti-oxidative enzyme paraoxonase (PON) or the anti-inflammatory protein apoAI. During an inflammatory state HDL may shed some of these components and take up other proteins such as serum amyloid A and oxidized lipids.\textsuperscript{43,57} The combination of these compositional alterations negatively affect the anti-atherosclerotic effect of HDL and may even result in the formation of pro-inflammatory HDL.\textsuperscript{58} Although it has already been shown in chronic inflammatory disorders such as SLE and RA that a significant proportion of HDL in these patients is proinflammatory,\textsuperscript{59} this remains to be evaluated in HIV-1 infection. HIV-1 infection may nonetheless directly
Atherosclerotic vascular disease in HIV

affect HDL functioning. The anti-atherogenic function of HDL is attributed to its role in reverse cholesterol transport (RCT), whereby excess cholesterol is transported from peripheral cells to HDL particles for subsequent delivery to the liver. The protein crucial for the initial step of RCT, is the ATP binding cassette transporter A1 (ABCA1). Recently it was shown that HIV-1 impairs ABCA1-dependent cholesterol efflux from human macrophages. The Nef protein, a protein encoded by one of accessory genes of HIV-1, induced post-transcriptional down-regulation of ABCA1 and caused redistribution of ABCA1 to the plasma membrane and inhibited internalization of apoAI. Infection with HIV-1 may thus impair removal of excess cholesterol resulting in augmented accumulation of cholesterol in macrophages, thereby accelerating the atherosclerotic process.

Endothelium

The endothelium constitutes the barrier that leukocytes need to cross prior to the formation of an atherosclerotic plaque. It is likely that the endothelium is affected by both HIV-1 infection itself as well as antiretroviral treatment. Indeed, cART may exert direct toxic effects on endothelial cells. For instance, it was shown that the HIV protease inhibitor ritonavir is able to directly cause endothelial mitochondrial DNA damage and cell death in vitro. HIV-1 itself may also exert direct atherogenic effects on the endothelium. HIV-1 can directly bind to the endothelium and reach the subendothelium by penetrating between endothelial cells via transcytosis. In addition, endothelial activation may also occur in HIV-1 infection either by cytokines secreted in response to mononuclear or adventitial cell activation by virus or else by the effects of the secreted HIV-1 proteins, gp120 (envelope glycoprotein) and Tat (transactivator of viral replication) on endothelium. Indeed, exposure of endothelial cells to gp120 results in increased expression of adhesion molecules and monocyte adherence which facilitates leukocyte recruitment to the subendothelium and thus atherosclerotic plaque growth. In addition, gp120 in the subendothelium may further facilitate atherothrombosis as it has been shown to activate human arterial smooth muscle cells to express tissue factor, the initiator of the coagulation cascade. Similarly, HIV-1 Tat has been shown to induce expression of adhesion molecules in endothelial cells and to increase the adhesion of monocytes and T-cells to the endothelium. Endothelial activation in HIV-1 infected patients has been confirmed by several studies showing increased levels of soluble adhesion molecules correlating with immunological state and progression of HIV-1 infection. In line with this, there is a relationship between deterioration of endothelial function in HIV-1 infected patients, as assessed by flow mediated dilation (FMD), and plasma HIV-1 RNA levels.

Coagulation

Under physiological conditions the endothelium protects against atherothrombosis by producing vasodilators such as nitric oxide and anti-coagulatory mediators. From the abovementioned endothelial dysfunction it can be deduced that the balance between stimulation and
inhibition of coagulation may also be disturbed. In a recent systematic review of ten epidemiological studies, the incidence of venous thromboembolic complications in HIV-1 infected patients was increased two- to tenfold compared with a HIV negative population of the same age.\textsuperscript{72} This hypercoagulable state in HIV-1 patients is suggested to result from a disturbed balance between increased procoagulatory (tissue factor, antiphospholipid antibodies, activated platelets) and reduced anticoagulant factors (protein S, protein C, antithrombin III, heparin cofactor II).\textsuperscript{73} These abnormalities correlate with severity of HIV-associated immunosuppression, as evidenced by CD4\textsuperscript{+} T-cell counts\textsuperscript{73} which might suggest that the higher immune activation that accompanies more severe immunodeficiency in HIV-1 infection underlies a hypercoagulable state in these patients. Although the mechanism of hemostatic changes thus appears to be a result of HIV-related triggering of the immune system, the coexistence of procoagulant conditions associated with HIV-1 infection such as superimposed (opportunistic) infections and malignancies, may also contribute to the hypercoagulable state.\textsuperscript{72,73}

5. HIV-1 and immunomodulation

The association between chronic immune activation and systemic inflammation in HIV-1 infection and acceleration of the atherosclerotic process is further strengthened by the observation that immunomodulation of HIV-1 can attenuate atherogenesis. For example, the chemokine receptor CCR5 plays an interesting role in this regard. A 32-bp deletion in the CCR5 gene (CCR5\textsuperscript{Δ32}) results in a nonfunctional receptor, and individuals that are homozygous for this deletion are not only resistant to infection with HIV-1\textsuperscript{74} but are also protected against atherosclerotic vascular disease.\textsuperscript{75} Interestingly, treatment with a CCR5 antagonist (TAK-779) was shown to not only exert potent and selective anti-HIV activity in Chinese hamster ovary cells\textsuperscript{76} but to also attenuate atherosclerotic lesion formation by blocking the influx of T-helper 1 cells into the plaque.\textsuperscript{77}

A second immunomodulatory agent with potential atheroprotective effects is mycophenolate mofetil (MMF), a produg of mycophenolic acid (MPA). MMF specifically inhibits lymphocyte proliferation\textsuperscript{78} and has been applied in studies in HIV-1 infected patients, since it is found to have both virological and immunological effects.\textsuperscript{79,81} Treatment with MMF resulted in a decrease of the reservoir of latently infected CD4\textsuperscript{+} T-cells.\textsuperscript{82} After discontinuation of MMF in patients treated with cART in another study,\textsuperscript{83} there was a temporary increase in Ki67 expression and apoptosis, indicating that MMF reduced the level of activation even in these patients. Interestingly, MMF has also been suggested to exert several anti-atherosclerotic functions\textsuperscript{84} and has been shown to reduce atherosclerotic lesion formation in animal studies.\textsuperscript{85,86} MMF inhibits the transfer of mannose and fucose to glycoproteins, some of which are adhesion molecules. MMF was shown to downregulate surface expression of adhesion molecules on endothelial cells and leucocytes and can therefore attenuate recruitment of circulating leukocytes to the
site of inflammation such as the atherosclerotic plaque. Two studies showed that New Zealand White rabbits which were fed a high cholesterol diet showed significantly larger areas of atherosclerotic plaques in the abdominal and thoracic aorta as compared to the MMF treated group. In the latter group, this reduction in atherosclerosis was associated with reduced number of macrophages and T-lymphocytes infiltrating the atherosclerotic plaque. The contention that immunomodulation in HIV-1 infection may not only be beneficial to attenuate HIV-mediated pathophysiology but also may translate into atheroprotection is an area of research that deserves further attention.

Figure 1 Schematic representation of enhanced atherogenesis in HIV

The use of cART is associated with insulin resistance, body fat changes and dyslipidemia. One regimen (ritonavir) has also been shown to induce cytotoxicity of human endothelial cells. Furthermore, exposure of endothelial cells to HIV-associated proteins results in increased expression of adhesion molecules and monocyte adherence. Moreover, HIV can penetrate the endothelium via transcytosis and has been shown to impair RCT and to activate human arterial smooth muscle cells (SMC) to express tissue factor (TF). Finally, systemic inflammation mediated by HIV-1 infection may also contribute to atherothrombotic disease by inducing dyslipidemia, activation of coagulation and endothelial dysfunction. It has been suggested that translocation of bacteria and bacterial components from the intestinal lumen, stimulate innate immune cells systemically, creating the proinflammatory milieu with inflammatory mediators (infl med) associated with chronic HIV infection.
6. Conclusion

The increased risk of atherosclerotic cardiovascular disease observed in patients on cART, and particularly on PI-containing cART, may be understood in terms of the dyslipidemia, insulin resistance and changes in body fat distribution associated with these treatments. However, HIV-1 infection by itself and by way of the chronic immune activation associated with the infection likely contributes to the risk of atherothrombotic disease through both direct and indirect mechanisms (see figure). Suppression of HIV replication by cART partly abrogates the state of chronic immune activation and may thus exert an atheroprotective effect. Further research is needed to unravel the role of these various treatment- and infection-associated factors towards the increased incidence of cardiovascular disease observed in HIV-1 infected patients in the era of cART. In the meantime prevention of cardiovascular disease by appropriately addressing traditional CVD risk factors in these patients is essential.
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7. The Strategies for Management of Antiretroviral Therapy (SMART) Study Group. CD4+ count-guided interruption of antiretroviral treatment. N Engl J Med. 2006; 355: 2283-2296. ** Large clinical trial which revealed that episodic antiretroviral therapy (guided by the CD4+ count) significantly increased both the risk of death from any cause, and the risk of fatal or nonfatal cardiovascular disease, as compared with continuous antiretroviral therapy.


* A comprehensive review of quality and function of high density lipoprotein

* Interesting study demonstrating that HIV-1 impairs ATP-binding cassette transporter A1 (ABCA1)-dependent cholesterol efflux from human macrophages


Effects of Nevirapine, Compared with Lamivudine, on Lipids and Lipoproteins in HIV-1 Uninfected Newborns: the Stopping Infection from Mother-to-Child via Breast-Feeding in Africa Lipid Substudy

Raaj R. Sankatsing
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Abstract

Objective: To assess whether the high-density lipoprotein cholesterol (HDL-c)-increasing effect of nevirapine (NVP), as observed in HIV-1 infected subjects, at least in part may relate to intrinsic properties of NVP.

Methods: At 2, 6 and 12 weeks after birth complete lipid profiles as well as plasma apolipoproteins levels were assessed in 80 HIV-uninfected newborns, half of whom received NVP and half lamivudine (3TC), respectively. Newborns were randomly selected from a randomized trial in which NVP or 3TC had been administered to HIV-uninfected infants born to HIV-infected mothers in order to try and prevent HIV-1 transmission from occurring during breastfeeding.

Results: Following six weeks of therapy the expected physiological decline in HDL-c levels in the newborns was attenuated in infants treated with NVP when compared to in those treated with lamivudine. Apolipoprotein A-I levels were higher at all time points in the NVP-arm as compared to the 3TC-arm (p=0.02) reaching peak levels at 6 weeks. The difference in HDL-c was no longer significant at 12 weeks.

Conclusions: apoA-I levels and HDL-c were elevated in HIV-1 uninfected newborns receiving NVP as compared to 3TC. These data support that NVP may indeed have intrinsic ApoA-I and HDL-c elevating properties in humans.
Introduction

The natural course of HIV infection has long been known to be associated with low levels of HDL-c. Previous studies have shown HDL-c levels to decrease by 24 to 37% after HIV seroconversion and prior to starting antiretroviral treatment [1,2], with a concomitant 27% decrease in apolipoprotein AI (ApoA-I), i.e. the major apolipoprotein of HDL [2]. Since HDL-c is an inverse phase reactant [3-6], first-time suppression of HIV by combination antiretroviral therapy (cART) can be expected to be accompanied by an increase in HDL-c, indicative of successful reversal of the pro-inflammatory state during active HIV infection. Indeed, first-time suppression of HIV with cART is associated with a 13% to 49% increase in HDL-c [7-13]. Since patients with more advanced HIV disease are characterized by lower HDL-c levels [14], a greater rise upon cART treatment is to be expected. Such an increase in HDL-c may in part be considered as a return towards normality.

Nonetheless, randomized clinical trials using different classes of cART regimens with similar potency for HIV suppression, found that regimens including the non-nucleoside reverse transcriptase inhibitor (NNRTI) nevirapine were associated with greater rises in HDL-c as well as apolipoprotein A-1 (apo-A1) compared to regimens including either the protease inhibitor indinavir [8,15] or nelfinavir [10]. Similarly, a trial comparing first-line cART regimens including either the NNRTI efavirenz or the protease inhibitor atazanavir found the former to be associated with a greater proportional rise in HDL-c, again in spite of similar antiviral efficacy [16,17]. Based on these results it has been suggested that both nevirapine and efavirenz may have intrinsic HDL-c elevating properties. In a head-to-head comparison of nevirapine and efavirenz-based first line cART regimens, both regimens indeed were confirmed to be associated with significant rises in HDL-c, with the rise being statistically significantly greater for nevirapine than efavirenz [7].

To further substantiate whether NNRTI’s intrinsically increase HDL-c levels, we evaluated the impact of nevirapine on HDL-c levels in the Stopping Infection from Mother-to-child via Breastfeeding in Africa (SIMBA) study. This study examined whether daily antiretroviral prophylaxis with either lamivudine (3TC) or nevirapine (NVP) administered to HIV-1 uninfected infants born to HIV-1 seropositive mothers could prevent postnatal mother-to-child-transmission (MTCT) of HIV-1. Although lipid profiles in neonates are fundamentally different from adults, this trial in HIV-uninfected individuals provided us with the unique opportunity to substantiate that the effect of nevirapine on HDL-c observed in HIV-infected persons receiving this drug at least in part may relate to intrinsic properties of nevirapine, rather than fully results simply from the suppression of the HIV infection. Thus, we performed a retrospective analysis of lipids and lipoproteins on stored plasma samples from a subset of uninfected newborns from the SIMBA study.
Figure 1  Simba trial flow chart

528 mothers screened for study entry
60 did not return or returned late
77 had abnormal lab values
16 delivered before enrolment
22 other reasons

413 mothers entered the study
4 lost to follow-up
2 serious adverse events (SAE)
1 patient withdrew

406 mothers, 414 pregnancy outcomes (including 8 twin pairs)
7 deaths shortly after birth (incl. 2 second born twins)
2 stillbirths
1 mothers’ request

3TC
202 infants

second born twins
excluded (HIV-1 uninfected mother)

199 liveborn first born infants

195 children ever breastfed (97%)

positive within 72 hours after birth
positive between day 4 and day 28
dead at day 25
lost to follow up before day 28

179 children at risk after 4 weeks

159 infants had normal completion

NVP
202 infants

3TC NVPRANDOMISATION

excluded (HIV-1 uninfected mother)

second born twins

198 liveborn first born infants

193 children ever breastfed (97%)

positive within 72 hours after birth
positive between day 4 and day 28
dead at day 25
lost to follow up before day 28

179 children at risk after 4 weeks

157 infants had normal completion

59 infants had normal completion

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Methods

This is a retrospective, comparative study to evaluate the effects of nevirapine and lamivudine on plasma lipids and lipoproteins in HIV-1 uninfected newborns. Eighty African newborns were randomly selected from the main SIMBA cohort for this analysis, 40 in each treatment arm. Only newborns that remained on the allocated treatment during the entire follow-up period were eligible for inclusion in the lipid substudy. In the main SIMBA study, 397 HIV-1 uninfected newborns (n=199 3TC, n=198 NVP), born from HIV-1 infected mothers in Rwanda and Uganda, were randomized in a 1:1 ratio to receive either NVP or 3TC from birth for the duration of breastfeeding plus an additional 4 weeks after stopping breastfeeding. This was done in order to prevent MTCT of HIV-1 through breastfeeding. Blood-samples from these newborn children were collected at baseline (that is, 2 to 3 days after birth) and 2, 6, 12 and 24 weeks after commencing the allocated treatment. Regretfully, no plasma samples were stored at baseline due to the limited amount of blood that can be drawn from newborns. The week 24 samples could also not be used because of insufficient available stored plasma samples. Consequently, only samples gathered at time points 2, 6 and 12 weeks post initiation of therapy were available and used for the current analysis. From the overall cohort of 397 newborn infants we determined the subcohort of newborns for whom both stored plasma samples were available at time points 2, 6 and 12 weeks after commencing treatment, and who had been continuously exposed to study medication during this time period. Newborns who either became infected during the treatment period, died, were lost to follow up or terminated the study on their mother’s request were not included in the subcohort (see figure 1). This selection resulted in a subcohort consisting of 316 newborns (157 in the NVP group and 159 in the 3TC group). The 80 newborns described in the current analysis were randomly selected from this subcohort. All assays for the current analysis were performed at the Academic Medical Center’s Laboratory for Experimental Vascular Medicine on plasma samples which had been cryopreserved at – 80 °C at the central laboratories.

Lipid analysis

Cholesterol concentrations in the main lipoprotein classes (very low-density lipoprotein (VLDL), LDL and HDL) were determined using high performance gel filtration chromatography (HPGC). The system contained a PU-980 ternary pump with an LG-980-02 linear degasser, FP-920 fluorescence and UV-975 UV/VIS detectors (Jasco, Tokyo, Japan). An extra P-50 pump (Pharmacia Biotech, Uppsala, Sweden) was used for in-line cholesterol PAP enzymatic reagent (Biomerieux, Marcy l’Etoile, France) addition at 0.1 ml/min. Plasma lipoprotein separations were performed with a Superose 6 HR 10/30 column (Pharmacia Biotech, Uppsala Sweden) with TBS pH 7.4, as eluent at a flow rate of 0.31 ml/min. Computer analysis of the chromatograms for quantification of the lipoproteins was carried out using Crompass Chromatographic software, version 1.7.403 (Jasco, Tokyo, Japan).
Commercially available lipid plasma standards (low, medium and high) were used for quantitative analysis (SKZL, Nijmegen, the Netherlands) for TC quantification. Apolipoprotein A-I (apoA-I) and apolipoprotein B (apoB) were both determined by nephelometric immunochemistry (Beckman, USA).

**Statistical analysis**
The changes over time in the measured lipid parameters were compared between the two randomization arms by a repeated measurements procedure using a generalized linear model (PROC MIXED of SAS software [SAS version 8.02, SAS Institute Inc, Cary, NC, USA]), which provides a valid statistical estimate of the mean effect. Such an analysis takes into account that serial measurements of the outcome variable in one patient are correlated. An unstructured covariate structure was used to give the best fit to the models. Categorical variables were compared between randomization arms using a chi-square test or Fisher's exact test where appropriate. Continuous variables other than the primary outcome variables were reported as medians plus interquartile range and were compared between randomization arms using the Wilcoxon two-sample test.

**Results**
The clinical characteristics of the newborns and their respective mothers are listed in table 1. The subset of infants randomly selected for the current analysis did not differ from the main cohort with regard to baseline characteristics. No significant differences were observed between the two groups of newborns in the current analysis with regard to Apgar scores, gender, and measurements associated with weight and infant size. Mean gestational ages between groups were also not different from each other. Also, no significant between-group differences were observed for the mothers with regard to age, weight, height, CD4+ T-cell count, plasma HIV-1 RNA levels and CDC classification at the time of delivery. Nutritional intake between the two groups is suggested to have been comparable as evidenced by nearly identical weight and height progression curves over the 12-week study period.

At 2, 6 and 12 weeks mean weights in the NVP-arm were 3.68 kg, 4.76 kg and 6.31 kg respectively, compared to 3.64 kg, 4.78 kg and 6.13 kg in the 3TC-arm.

**Lipids**
Changes in lipids and lipoproteins in the course of the study are summarized in table 2. Total cholesterol (TC) (normal values at birth: \(1.86 \pm 0.41\) mmol/L [18]) increased from 3.20 mmol/L at week 2 to 3.83 mmol/L at week 12 in the NVP-arm, whereas in the 3TC-arm it increased less from 3.24 to 3.49 mmol/L (p=0.025).
Table 1  Clinical characteristics of newborns and their mothers at time of delivery

<table>
<thead>
<tr>
<th></th>
<th>NVP (n=40)</th>
<th>3TC (n=40)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Child parameters</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gestational age (weeks)</td>
<td>40.3 (39.3-41.3)</td>
<td>40.3 (39.1-41.3)</td>
<td>0.86</td>
</tr>
<tr>
<td>Apgar score 1'</td>
<td>10 (8-10)</td>
<td>10 (8-10)</td>
<td>0.51</td>
</tr>
<tr>
<td>Apgar score 5'</td>
<td>10 (10-10)</td>
<td>10 (10-10)</td>
<td>0.10</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>50.0 (49.0-50.5)</td>
<td>50.0 (48.0-51.0)</td>
<td>0.67</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>3.2 (2.9-3.5)</td>
<td>3.2 (3.0-3.4)</td>
<td>0.60</td>
</tr>
<tr>
<td>Head circumference (cm)</td>
<td>35 (34-36)</td>
<td>35 (34-36)</td>
<td>0.63</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>13.1 (11.6-13.7)</td>
<td>12.8 (11.9-13.8)</td>
<td>0.78</td>
</tr>
<tr>
<td>Male gender (n [%])</td>
<td>19 (47.5)</td>
<td>22 (55)</td>
<td>0.65</td>
</tr>
<tr>
<td><strong>Maternal parameters</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>27 (24-30)</td>
<td>27 (25-31)</td>
<td>0.48</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>64.0 (59.2-69.5)</td>
<td>63.0 (59.5-67.6)</td>
<td>0.82</td>
</tr>
<tr>
<td>Length (cm)</td>
<td>160.0 (153.5-164.0)</td>
<td>159.5 (156.0-163.5)</td>
<td>0.90</td>
</tr>
<tr>
<td>CD4+ T-cell count (cells/mm³)*</td>
<td>467 (380-658)</td>
<td>421 (294-616)</td>
<td>0.33</td>
</tr>
<tr>
<td>Plasma HIV viral load</td>
<td>2.60 (2.60-2.87)</td>
<td>2.64 (2.60-3.29)</td>
<td>0.13</td>
</tr>
<tr>
<td>(log10 copies/mL)³</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Undetectable plasma viral load (%)†</td>
<td>67.5</td>
<td>50</td>
<td>0.17</td>
</tr>
<tr>
<td>CDC classification (n [%])</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>38 (95)</td>
<td>39 (97.5)</td>
<td>1.00</td>
</tr>
<tr>
<td>B</td>
<td>2 (5)</td>
<td>1 (2.5)</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td></td>
</tr>
</tbody>
</table>

Data are presented as median [interquartile range] unless indicated otherwise. BMI indicates body mass index.

* Maternal CD4+ T-cell count collected at 36 weeks of gestation.

§ Maternal HIV viral load measured at delivery.

† Plasma HIV viral load < 400 copies per milliliter.

The net mean change in HDL-c (normal values at birth: 0.88±0.23 mmol/L [18]) between week 2 and week 12 was -0.47 mmol/L in the NVP-arm compared to -0.57 mmol/L in the 3TC-arm (Fig. 2). Overall, the HDL-c curves in the two arms were not statistically different (p=0.17). However, there was a highly significant interaction between treatment and time (p=0.0029). In the NVP arm mean HDL-c increased initially up to 1.58 mmol/L at week 6 and decreased thereafter, whereas it decreased consistently in the 3TC-arm. The net mean change in HDL-c between week 2 and week 6 was +0.11 mmol/L in the NVP arm compared to -0.19 mmol/L in the 3TC arm. To compare the HDL-c levels between the two arms at week 6, we employed the ‘slice’ option of the LSMEANS statement of PROC MIXED from SAS, and found a significant difference (p=0.012). There were no statistically significant differences in changes over time in LDL-c (normal values at birth: 0.75±0.34 mmol/L [18]) (p=0.61). LDL-c increased from 1.29 mmol/L at week 2 to 1.98 mmol/L at week 12 in the NVP-arm. A similar increase was observed in the 3TC-arm (from 1.35 to 1.92 mmol/L). VLDL-c increase was significantly greater in the NVP-arm compared to the 3TC-arm between week 2 and week 12 (0.44 mmol/L to 0.85 mmol/L vs. 0.40 mmol/L to 0.64 mmol/L, respectively; p=0.006).
Apolipoproteins
The changes over time in ApoA-I (normal values at birth: 770±130 mg/L [18]) were significantly different between the two arms (p=0.02). ApoA-I increased by 229 mg/L between week 2 and week 6 in the NVP-arm (from 1355 mg/L to 1584 mg/L) compared to 203 mg/L in the 3TC-arm (from 1265 mg/L to 1468 mg/L). In line with the pattern of HDL-c, apoA-I levels decreased by 176 mg/L in the NVP-arm and 190 mg/L in the 3TC arm between week 6 and week 12. The absolute mean change in apoA-I between week 2 and week 12 was +53 mg/L in the NVP-arm as compared to +13 mg/L in the 3TC-arm (Figure 2). The changes over time in ApoB (normal values at birth: 280±90 mg/L[18]) were not significantly different between the two arms (p=0.10). ApoB increased steadily in both arms, but the net mean change in the NVP-arm was not different from that of the 3TC-arm (+400 mg/L vs. +361 mg/L, respectively). In agreement with these findings, the calculated apoB/apoA-I ratio increased over the course of the study in both treatment arms.

Table 2 Changes of lipids and lipoproteins over time for NVP and 3TC-treated newborns

<table>
<thead>
<tr>
<th>Variable</th>
<th>NVP (n=40)</th>
<th>3TC (n=40)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wk 2</td>
<td>Wk 6</td>
<td>Wk 12</td>
</tr>
<tr>
<td>TC (mmol/L)</td>
<td>3.20±0.09</td>
<td>3.71±0.11</td>
<td>3.83±0.11</td>
</tr>
<tr>
<td>HDL-c (mmol/L)</td>
<td>1.47±0.07</td>
<td>1.58±0.07</td>
<td>1.00±0.05</td>
</tr>
<tr>
<td>LDL-c (mmol/L)</td>
<td>1.29±0.06</td>
<td>1.61±0.07</td>
<td>1.98±0.07</td>
</tr>
<tr>
<td>VLDL-c (mmol/L)</td>
<td>0.44±0.03</td>
<td>0.52±0.03</td>
<td>0.85±0.05</td>
</tr>
<tr>
<td>ApoA-I (mg/L)</td>
<td>1355±36</td>
<td>1584±48</td>
<td>1408±40</td>
</tr>
<tr>
<td>ApoB (mg/L)</td>
<td>561±27</td>
<td>683±25</td>
<td>961±31</td>
</tr>
<tr>
<td>ApoB/ApoA-I ratio</td>
<td>0.41</td>
<td>0.43</td>
<td>0.68</td>
</tr>
</tbody>
</table>

Data are presented as modeled means ± the standard error of the mean except for the ApoB/ApoA-I ratio. The reported p-value is from the type 3 test of fixed effects, comparing the overall difference of the profile of the lipid parameter of interest between the NVP and 3TC arm. Wk indicates week; TC, total cholesterol; HDL-c, high density lipoprotein cholesterol; LDL-c, low density lipoprotein cholesterol; VLDL-c, very low density lipoprotein cholesterol; TG, triglycerides; ApoA-I, apolipoprotein A-I and apoB, apolipoprotein B.

Discussion
In the present study we show that infants, in the absence of HIV-1 infection, following exposure to NVP as compared to 3TC, had higher HDL-c and apo-A1 levels after 6 weeks of treatment. These results imply that NVP may indeed exert an intrinsic effect on apoA-I and HDL metabolism, which concurs with earlier studies in HIV-1 infected adults [7,8,10,15].

NVP and apoA-I/HDL-c increase
At first glance, it is apparent that the HDL-c increase in these infants is modest compared to increases up to 49% reported upon initiation of NVP in HIV-1 infected adults [8]. The latter most likely reflects the fact that resolution of the pro-inflammatory state upon NVP initiation in
HIV-1 infected adults provides a potent stimulus for HDL-c increase, which is obviously absent in HIV-1 negative newborns. In support, studies in HIV-1 infected adults switching to NVP after HIV-1 infection had first been suppressed with other antiretroviral regimens, also reported lower HDL-c increases compared to studies in ART-naïve patients starting nevirapine-containing ART [19,20].

Upon interpreting HDL-c changes after birth, it is mandatory to take into account the physiological changes of HDL-c in healthy babies. At the time of birth, HDL-c levels are approximately 60% of adult levels, i.e. 0.80 mmol/L [21-25]. Within the first month of life, HDL-c levels show a strong increase [26], which is followed by a steady decrease in the second and third months [27,28]. This decrease is predominantly due to increasing triglyceride levels, which induce the transfer of cholesterol ester from HDL-c to VLDL-c via the enzyme cholesteryl ester transfer protein (CETP), resulting in lower HDL-c levels [29]. Unfortunately, HDL-c levels at the time of birth were not available in our cohort. Nonetheless, there was a significant increase in HDL-c in the NVP group between week 2 and 6, whereas HDL-c levels already declined in the 3TC group. In line with HDL-c levels, apoA-I increase was also higher in the NVP group compared to the 3TC group. Whereas the difference in HDL-c levels was no longer significant at week 12, apo-A-I levels remained significantly higher in infants exposed to NVP at week 12. A potential explanation contributing to loss of HDL-c but not apoA-I increase at week 12 could relate to a shift from mature α-HDL particles to smaller nascent pre-β HDL particles in the NVP group. Given the presumed greater free cholesterol acceptor capacity of pre-β HDL this change might be beneficial. However, given the limited data available we can only speculate on the nature of this observation. The underlying mechanism for the increase in apoA-I could either be the effect of an increased production or decreased catabolism of apoA-I under the influence of NVP. However, since our study did not set out to investigate this we cannot substantiate either option here. Theoretically, there are 2 options explaining the HDL-c patterns observed. On the one hand, NVP may have intrinsic HDL-c increasing capacity; on the other hand, 3TC may have detrimental effects on HDL-c with the HDL profile in the NVP-treated newborns merely following the natural course of HDL-c after birth. With respect to the latter option, increased catabolism of HDL-c upon 3TC treatment most likely pertains to the concomitant increase in VLDL-c, which induces exchange of cholesterol from HDLc via the CETP pathway [29]. In the present cohort, however, VLDL-c levels were actually lower in the 3TC group compared to the NVP group, rendering this a less likely explanation for the differences observed between the two treatment groups. With respect to NVP having intrinsic HDL-c increasing capacity, potential underlying mechanisms include changes in the activity of HDL-modifying enzymes such as lipoprotein lipase, lecithin:cholesterol acyl transferase or cholesteryl ester transfer protein as well as increased apoA-1 synthesis. Whereas the present study does not provide us with mechanistical clues, such studies are currently ongoing in HIV-1 infected adults.
Figure 2  Course of cholesterol and apolipoproteins

A  HDL cholesterol

mmol/L

0  0.5  1.0  1.5  2.0

Weeks since birth

3TC  NVP

B  ApoA-I

mg/L

1000  1100  1200  1300  1400  1500  1600

Weeks since birth

3TC  NVP

C  LDL cholesterol

mmol/L

0  0.5  1.0  1.5  2.0

Weeks since birth

3TC  NVP

D  ApoB

mg/L

400  500  600  700  800  900  1000

Weeks since birth

3TC  NVP

E  VLDL cholesterol

mmol/L

0.00  0.25  0.50  0.75  1.00

Weeks since birth

3TC  NVP

Weeks indicates the duration of therapy from birth onwards. Solid circles represent newborns treated with nevirapine, open circles represent 3TC-treated newborns.

* The difference between NVP and 3TC is significant at the week 6 time point in the HDL graph (p=0.011).
**NVP and VLDL-c increase**

In line with expectation, a rise in triglyceride-rich lipoproteins (VLDL-c) was observed in both treatment groups. The latter reflects increased capacity to absorb dietary fatty acids, which are secreted at the level of the liver as VLDLs. In parallel to VLDL-c increase, its structural protein apoB also increases progressively. The increase in VLDL-c was higher in the NVP-arm as compared to the 3TC-arm. Several explanations can be envisaged for this phenomenon. Increased VLDL-c levels can be caused by either increased production of VLDL-c or by decreased removal of VLDL-c by lipoprotein lipase, i.e. the principal enzyme mediating enzymatic VLDL metabolism. Whereas we cannot distinguish between increased VLDL synthesis or decreased VLDL clearance associated with NVP use, studies in adult HIV patients have not substantiated VLDL-c increases during NVP use [20]. Another option is attenuation of the physiological VLDL-c increase in the 3TC group as a consequence of decreased uptake of dietary fats due to gastrointestinal side-effects of 3TC. However, the latter is not substantiated by identical weight curves in time for both treatment arms. Notably, the larger VLDL-c increase in the NVP-arm will stimulate CETP-mediated transfer of cholesterol esters from HDL-c to VLDL-c [30]. Hence, this may have contributed to attenuation of the HDL-c increase in the NVP group at 12 weeks. LDL-c levels showed a physiological increase which was similar in both treatment groups.

**Study limitations**

Our study has several limitations, which includes the lack of lipid measurements at birth. In view of the absence of baseline measurements, it could be postulated that the observed differences between the treatment arms merely reflect differences which were already present at birth. However, the latter is unlikely since treatment was successfully allocated in a random fashion as illustrated by good comparability of baseline characteristics between the two treatment groups. Furthermore, interpretation of the lipid changes with time is hampered by the absence of an untreated control arm. However, the inclusion of an untreated control group was deemed unethical at the time the trial was designed from the point of view of the primary aim of the trial which was to prevent MTCT of HIV-1 during breastfeeding. Thus, we had to refer to historical control values for lipid changes after birth which we derived from published cohorts.

In conclusion, the results from our study in infants exposed to NVP in the absence of HIV-1 infection suggest that rises in HDL-c and apoA-I which were previously reported in HIV-1 infected adults treated with NVP-containing ART result, at least in part, from an intrinsic property of this drug. Whether this property of NVP may modify the risk of cardiovascular events in HIV-1 infected individuals treated with cART remains to be determined.
Acknowledgements

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Reference List


Increased Carotid Intima-Media Thickness in HIV patients treated with Protease Inhibitors as compared to Non-Nucleoside Reverse Transcriptase Inhibitors

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John JP Kastelein
Erik SG Stroes
Peter Reiss

Abstract

**Background:** Prolonged exposure to protease inhibitor (PI)-, but not non-nucleoside reverse transcriptase inhibitor (NNRTI)-containing combination antiretroviral therapy (CART) has been associated with an increased cardiovascular risk, partly explained by the different effects of these drugs on plasma lipids. Most markedly, NNRTIs have been associated with increases in high density lipoprotein cholesterol (HDL-C), which may be atheroprotective.

**Methods:** In a cross-sectional study we investigated the impact of PI- versus NNRTI-based CART in 130 HIV-1-infected patients with plasma virus suppressed to below the limit of detection, whom had been continuously exposed for at least 2 years to either one of such regimens, but not both. Carotid intima-media thickness (C-IMT) and fasting metabolic parameters were measured.

**Results:** Mean (±SD) C-IMT in patients treated with PI-based CART was 0.81 (±0.17) mm as compared to 0.71 (±0.14) mm in NNRTI treated patients (p=0.0003). HDL-C and apolipoprotein A-I (apoA-I) levels were higher in the NNRTI than in the PI group (1.39 versus 1.03 mmol/L, p<0.0001, and 1.44 versus 1.33 mmol/L, p=0.0008, respectively). Age, body mass index, duration of CART, and use of PI-based CART were positively correlated with C-IMT whereas HDL-C and apoA-I were inversely correlated with C-IMT.

**Conclusions:** Treatment of HIV-1-infected patients for two years or more with PI-based compared to NNRTI-based CART is associated with greater C-IMT, consistent with the reported higher risk of CVD in patients using PI. However, this difference seems not fully explained by a more favorable impact of NNRTI-based CART on HDL-C and apoA-I levels.
Introduction

AIDS-related mortality was profoundly reduced when combination antiretroviral therapy (CART) was introduced into clinical practice. However, CART use was also found to cause a range of metabolic complications, including insulin resistance and diabetes mellitus, as well as dyslipidemia and changes in body fat distribution or lipodystrophy.\textsuperscript{1,2} This has fueled the concern that patients using CART might be at increased risk of cardiovascular disease (CVD). The largest study that prospectively addressed this question, the Data Collection on Adverse Events of Anti-HIV Drugs (D:A:D) Study, indeed revealed that CART, independent of traditional CVD risk factors, was associated with an increased risk of myocardial infarction.\textsuperscript{3} In a recent update from this study, the risk of myocardial infarction associated with CART proved to be confined to protease inhibitors.\textsuperscript{4} Conversely, no evidence was found for such an association with non-nucleoside reverse transcriptase inhibitors. This association with PI exposure could only partly be explained by the dyslipidemia seen with CART.

HIV treatment that includes protease-inhibitors (PIs), in particular when boosted by ritonavir, is often associated with increased plasma levels of triglycerides (TG) and low density lipoprotein (LDL)-cholesterol, with modest or no consequences for high density lipoprotein cholesterol (HDL-C). These effects on plasma lipids are much less pronounced when non-nucleoside reverse transcriptase inhibitors (NNRTIs) such as nevirapine or efavirenz are used.\textsuperscript{5-8} Of note, in contrast to PIs, NNRTIs have been associated with marked increases in HDL-C.\textsuperscript{5-9} The latter is of particular interest since high HDL-C may protect against CVD due to its ability to promote reverse cholesterol transport\textsuperscript{10} and a range of additional mechanisms which include antioxidative and anti-inflammatory properties.\textsuperscript{11}

We therefore speculated that NNRTI-containing CART, in contrast to regimens that include protease inhibitors, would be associated with a slower onset of atherosclerotic vascular disease. In order to test this hypothesis, we performed a cross-sectional study in which we compared the carotid intima-media thickness (C-IMT) in HIV-1-infected patients with viral suppression below the limit of detection, who had continuously been exposed for two years or longer to either PI- or NNRTI-containing CART, but not both.
Methods

Patient Selection
All eligible patients who consecutively attended the HIV outpatient clinics of the Academic Medical Center and the Onze Lieve Vrouwe Gasthuis in Amsterdam, the Netherlands, as well as of the Universitätsklinikum in Bonn, Germany between June 2003 and February 2006, and who met the inclusion criteria for the study were approached to participate. Patients could be included if they were on either PI- or NNRTI-based CART and had been receiving such a regimen continuously for at least two years. Furthermore patients had to have documented plasma HIV-1 RNA levels to below the limit of detection (< 50 copies/mL) at the time of study entry. Patients in the PI-treated group were allowed to have switched between different PIs, but could not have been exposed to NNRTI while patients in the NNRTI-group had to have been treated with either nevirapine- (NVP) or efavirenz- (EFV) containing CART, and were not allowed to have switched between these two NNRTIs, or to have ever used PIs. The study protocol was approved by the institutional review board of the Academic Medical Center, University Hospital of Amsterdam. Patients from the Onze Lieve Vrouwe Gasthuis in Amsterdam were referred for the study to the Academic Medical Center. No ethics approval was needed for patients from Bonn according to local guidelines, given that no intervention was involved and all bloods were drawn according to routine clinical care. All subjects provided written informed consent.

Cardiovascular Risk Factor Assessment
At the study visit a detailed medical history was obtained from each patient using a standardized questionnaire including questions concerning demographic characteristics, prior and current antiretroviral treatment, family history of CVD, prior history of CVD, cigarette smoking, and use of antihypertensive, antidiabetic, or lipid lowering drugs. Patients’ height and weight, as well as blood pressure in supine position were measured in a standardized fashion. Framingham Risk Scores were calculated for each patient.

In addition, at the same visit blood was drawn following an overnight fast for assessment of total- and HDL cholesterol (HDL-c), triglycerides (using enzymatic methods, Roche Diagnostics GmbH, Mannheim, Germany), apolipoprotein A-I (apoA-I), apolipoprotein B (apoB) (using rate immunonephelometry, Dade Behring Nephelometer BNII, Marburg, Germany), high-sensitivity C-reactive protein (hs-CRP) (using an immune turbidimetric test [Roche Diagnostics GmbH, Mannheim, Germany]), aspartate aminotransferase, alanine aminotransferase (using the Pyridoxalphosphate, 37 °C UV test [Roche Diagnostics GmbH, Mannheim, Germany]), glucose (using the HK/Glucose-6-P dehydrogenase UV test [Roche Diagnostics GmbH, Mannheim, Germany]), free T4 and thyroid stimulating hormone (TSH) (using a solid phase, two-site fluor-immunometric assay [PerkinElmer Life and Analytical Sciences, Turku, Finland]). LDL cholesterol (LDL-c) was calculated using the Friedewald equation. Finally, in Amsterdam absolute and percent CD4- and CD8-positive lymphocyte subsets were determined by flow cytometry (BD
Multitest CD3FITC/CD8PE/CD45PerCP/CD4APC [BD Biosciences, San Jose, USA], and plasma HIV-RNA was measured using the Versant HIV-1 RNA 3.0 assay (bDNA)(Siemens Medical Solutions Diagnostics, Los Angeles, USA) with a lower limit of detection of 50 copies/mL.

In Bonn, HIV-RNA was measured using bDNA assay (Bayer Vital GmbH Diagnostika, Fernwald, Germany), likewise with a lower limit of detection of 50 copies/mL, while CD4- and CD8-cell counts were analyzed on a FACS calibur flow cytometer (Becton Dickinson, Heidelberg, Germany). Hepatitis B and C co-infection was not assessed.

Measurement of Carotid Intima-Media Thickness

The common carotid, carotid bulb, and internal carotid arterial far walls were scanned bilaterally using a standardized B-mode ultrasound imaging and image analysis protocol, the details of which have been described previously elsewhere. In summary, an Acuson 128XP ultrasound instrument (Acuson, Mountainview, CA, USA) equipped with an L7 transducer and Extended Frequency (EF) software was used. Standard views of 2 by 2 centimeters were obtained and saved as 4:1 compressed JPEG image files. For off-line image analyses in-house designed validated hardware and software was used. A subject’s carotid IMT was calculated as the average of the sum of the 3 right and 3 left carotid arterial far wall IMT measurements. Two experienced sonographers, blinded to the subject’s antiretroviral regimen, scanned all subjects both in the Netherlands and in Germany. A technician, who remained blinded to each patient’s CART history, subsequently performed the off-line image analyses.

Statistical methods

Baseline parameters were tabulated and compared between groups using Fisher’s exact or Wilcoxon test as appropriate. The C-IMT was compared among groups using Student’s t-tests. Univariate and multivariate linear regression was used to investigate the influence of selected covariates on the C-IMT. Covariates that were found to be statistically significantly associated with C-IMT in a univariate analysis were entered into the multivariate analyses. The final multivariate linear regression model only contained covariates that remained significantly associated with the C-IMT. Investigated covariates were: (duration of) used antiretroviral agents, (duration of) smoking, blood lipids and glucose and CRP, presence of diabetes mellitus, age, gender, blood pressure, BMI, weight, and the Framingham Risk Score. The sample size was calculated assuming a difference in IMT favouring patients using NNRTI- compared to PI-based therapy of at least 0.05 mm over 2 years with a standard deviation of 0.1. The study was originally designed to compare 40 patients using nevirapine with 80 patients using protease inhibitors, which would yield 80% power using single-sided significance testing. Before the start of the study the design was changed to also include 40 patients using efavirenz. We assumed similar findings in the NVP and EFV subgroups. The main comparison was now between the PI (n=80) and the combined NNRTI group (n=80), which had 88% power using 2-sided significance testing. Eventually we were able to recruit just 62 and 68 patients into the PI and the
combined NNRTI groups, yielding 81% power using 2-sided testing. The study was not powered
to formally compare between the EFV and NVP subgroups. Statistical significance was set at
a 2-sided p-value of 0.05 for all comparisons. All analyses were done using SAS software (SAS
version 9.1, SAS institute, Cary, NC, USA).

Results

Patient characteristics

A total of 130 HIV-1-infected patients were enrolled in this study, of whom 62 were receiving PI-
based CART and 68 NNRTI-based CART. Within the NNRTI-group, 38 patients were on NVP and
30 patients on EFV. The clinical characteristics of the patients are listed in table 1. The median
age of the total cohort was 46 years, and 90% were male.

The groups of patients on PI- and NNRTI-based CART were comparable with respect to age,
sex, body mass index, history of CVD, presence of current other risk factors for CVD, and con-
comitant use of antihypertensive, antidiabetic and lipid lowering medication. No differences
were found in Framingham Risk Scores between NNRTI and PI-treated patients.

A specification of PI use is provided in table 2.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Clinical characteristics of patients on PI- and NNRTI-based CART</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PI (n=62)</td>
</tr>
<tr>
<td>Age, y</td>
<td>45 (40-54)</td>
</tr>
<tr>
<td>Sex, female (%)</td>
<td>5 (8)</td>
</tr>
<tr>
<td>Body Mass Index, kg/m²</td>
<td>23 (21-25)</td>
</tr>
<tr>
<td>History of CVD, n (%)</td>
<td>6 (10)</td>
</tr>
<tr>
<td>Risk factors CVD, n (%)</td>
<td></td>
</tr>
<tr>
<td>Current Smoking</td>
<td>27 (44)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>7 (11)</td>
</tr>
<tr>
<td>Diabetes Mellitus</td>
<td>2 (3)</td>
</tr>
<tr>
<td>Family history premature CVD</td>
<td>4 (6)</td>
</tr>
<tr>
<td>Current Medications, n (%)</td>
<td></td>
</tr>
<tr>
<td>Antihypertensive drugs</td>
<td>8 (13)</td>
</tr>
<tr>
<td>Antidiabetic drugs</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Lipid lowering drugs</td>
<td>7 (11)</td>
</tr>
<tr>
<td>Pack-years of smoking, y</td>
<td>21 (14-30)</td>
</tr>
<tr>
<td>SBP, mmHg</td>
<td>123 (119-135)</td>
</tr>
<tr>
<td>DBP, mmHg</td>
<td>78 (72-84)</td>
</tr>
<tr>
<td>Pulse pressure, mmHg</td>
<td>48 (42-54)</td>
</tr>
<tr>
<td>Duration of CART use (y)</td>
<td>5.12 (3.99-8.86)</td>
</tr>
<tr>
<td>Framingham Risk Score</td>
<td>6 (3-12)</td>
</tr>
</tbody>
</table>

CVD denotes cardiovascular disease, SBP systolic blood pressure, DBP diastolic blood pressure, and CART combination antiretroviral therapy. Current smoking was defined as smoking within the last month.
Results of laboratory assessments (Table 3)

As per protocol all patients had a plasma HIV-1 RNA level below the limit of detection of 50 copies per milliliter. The median CD4- and CD8-positive lymphocyte counts and the CD4/CD8 ratio were not significantly different between PI- and NNRTI-treated patients. Mean HDL-C levels were significantly higher in the NNRTI-group than in the PI group (1.39 (1.16-1.66) mmol/L, respectively; p<0.0001). Within the NNRTI-group, they were somewhat higher in the NVP subgroup compared to the EFV subgroup (1.44 (1.26-1.80) mmol/L vs. 1.37 (1.03-1.54) mmol/L, p=0.021).

Likewise apolipoprotein A-I (apoA-I) levels were significantly higher in the NNRTI group compared with the PI group (1.44 [1.30-1.56] mmol/L vs. 1.33 [1.16-1.45] mmol/L, p=0.0008), whereas triglyceride levels were lower in the NNRTI group (1.2 [1.0-1.7] mmol/L vs. 2.1 [1.4-3.4] mmol/L, p<0.0001). Glucose levels were within the normal range in both groups, but somewhat higher in the NNRTI group as compared to the PI group (5.1 [4.7-5.4] mmol/L vs. 4.6 [4.3-5.2] mmol/L, p=0.0016).

No significant differences were observed in levels of C-reactive protein, liver aminotransferases and thyroid hormones.

Carotid IMT

Mean (±SD) C-IMT was 0.81 (±0.17) mm in the PI-treated group compared to 0.71 (±0.14) mm in the NNRTI-treated group (p=0.0003) (Figure 1). In view of the fact that we had a reasonably substantial number of patients on NVP and EFV respectively, a post-hoc exploratory analysis was performed comparing each of these subgroups to the patients on PI. In this comparison both the NVP-subgroup as well as the EFV-subgroup showed thinner C-IMT as compared to the PI-group, 0.72 (±0.13) mm (NVP) vs. 0.81 (±0.17) mm (PI) (p=0.0031), and 0.70 (±0.15) mm (EFV) vs. 0.81 (±0.17) mm (PI) (p=0.0027). On univariate analyses, increasing age, systolic and diastolic blood pressure, BMI, pack years of smoking, triglycerides, apoB levels, duration of CART use, Framingham Risk Score, as well as use of any PI and use of ritonavir in particular, were associated with increased C-IMT. In contrast, increasing levels of HDL-C and apoA-I were associated with decreased C-IMT.

Of those parameters which were univariately associated with C-IMT, only age (+0.05 mm per 10 years older), BMI (+0.011 mm per 1 kg/m² increase), duration of CART use (+0.011 mm per additional year of exposure) and the use of PI (+0.10 mm for PI use) remained significantly and independently associated with C-IMT on multivariate analysis (Table 4). A trend remained for diastolic blood pressure (+0.025 mm per 10 mmHg increase) on multivariate analysis.
Figure 1  C-IMT of HIV-infected patients on PI- and NNRTI-based CART vs. PI

C-IMT is significantly thicker in patients using PI compared to NNRTI.
Data are presented as mean ± standard deviation. * p<0.001 NNRTI vs. PI

Discussion

Our results show that exposure of HIV-1-infected adults for two years or more to PI-containing, but not NNRTI-containing CART, was significantly associated with increased C-IMT, independent of traditional cardiovascular risk factors. These findings are of interest, because increases in C-IMT are predictive of future cardiovascular events. C-IMT is regarded a validated surrogate marker for cardiovascular disease, and because of recently published results from the D:A:D Study which reported an increased risk of myocardial infarction with PI but not with NNRTI use. Interestingly, the association between the odds for myocardial infarction and PI exposure could only partly be explained by the dyslipidemic effects of therapy, suggesting that additional factors related to the use of PI are contributing to this risk.

Table 2  Specification of protease inhibitors used by PI treated patients

<table>
<thead>
<tr>
<th>Protease inhibitor</th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>APV+LPV/r</td>
<td>1 (1.61)</td>
</tr>
<tr>
<td>APV/r</td>
<td>4 (6.45)</td>
</tr>
<tr>
<td>ATZ/r</td>
<td>3 (4.84)</td>
</tr>
<tr>
<td>IDV</td>
<td>5 (8.06)</td>
</tr>
<tr>
<td>IDV/RTV</td>
<td>4 (6.45)</td>
</tr>
<tr>
<td>LPV/r</td>
<td>34 (54.84)</td>
</tr>
<tr>
<td>NFV</td>
<td>3 (4.84)</td>
</tr>
<tr>
<td>RTV (full dose)</td>
<td>4 (6.45)</td>
</tr>
<tr>
<td>SQV/RTV</td>
<td>4 (6.45)</td>
</tr>
<tr>
<td>Total</td>
<td>62 (100)</td>
</tr>
</tbody>
</table>

Footnote: APV denotes amprenavir, LPV lopinavir, ATZ atazanavir, IDV indinavir, NFV nelfinavir, RTV “full” dose ritonavir (i.e. 400-600 mg twice daily), SQV saquinavir, and r boosting dose of ritonavir (i.e. 100-200 mg twice daily)
Classical risk factors for CVD were overall balanced between the two groups with no parameter being significantly different between groups. Although in our study levels of HDL-C and apoA-I were significantly higher in the NNRTI-treated patients, and inversely related to C-IMT in the univariate analysis, this association no longer remained significant in the multivariate analysis. The latter only revealed PI use, duration of CART use, age, and BMI as significantly associated with C-IMT. However, given the strong univariate associations between PIs and IMT on the one hand and HDL and IMT on the other hand as well as the association between NNRTI and HDL, it is difficult to identify an “independent” effect of NNRTI above and beyond HDL. Of note, previous studies have shown increases in HDL-C levels both in therapy-naïve patients commencing treatment with nevirapine- or efavirenz-based CART, as well as in patients in whom nevirapine or efavirenz was substituted for a PI. In general HDL-C increases in these studies have been more pronounced with nevirapine- than efavirenz-containing CART. Although the latter was confirmed in the current study, HDL-C levels were significantly higher for patients on either NVP or EFV when compared to those on PIs. Comparison of the individual NNRTIs with PIs showed consistent results with respect to C-IMT although this analysis has to be interpreted with caution in view of more limited power. Contrary to what would be expected, we found similar levels of LDL-C in PI- and NNRTI-treated patients and slightly lower levels of glucose in PI-treated patients. While these observations may reflect inadequate matching previous studies show that, in contrast to NVP, EFV increases LDL-C levels which may have obscured a possible difference in LDL-C levels between the two groups.

It is important to realize however that, in addition to any antiretroviral treatment-associated effect not mediated by lipid changes, factors related to the HIV-1 infection itself as well as the state of immunodeficiency and/or immune activation associated with the infection may also contribute to atherogenesis and cardiovascular disease in the context of HIV-1 infection. Results from the SMART (Strategies for Management of Antiretroviral Therapy) trial have raised the awareness for this concept. In this trial the intermittent use of antiretroviral therapy guided by patients’ CD4⁺ lymphocyte counts (drug conservation strategy) was associated with a higher rate of fatal or nonfatal cardiovascular events compared to when antiretroviral therapy was used continuously (viral suppression strategy). These increased odds in the drug conservation arm were highest for those already entering the trial off antiretroviral therapy and to a lesser degree for those entering on therapy and discontinuing NNRTI-containing therapy. Lipid changes, including reductions in HDL-C and increased total/HDL-C ratios, were also more unfavorable in patients allocated to drug conservation. This was particularly the case in those on NNRTI at entry, which may partially explain the observed excess CVD risk in the drug conservation strategy arm. Thus, multiple factors both related and unrelated to antiretroviral therapy, likely act in concert in promoting atherogenesis in patients with HIV-1 infection. This renders it difficult to disentangle the individual contribution of any one of these factors, including the effect of particular antiretroviral drug classes. This difficulty is also illustrated by the results of
previous studies examining C-IMT in patients with HIV-1 infection. Several but not all previous studies which included an HIV-uninfected control group have demonstrated the presence of HIV-1 infection to be associated with increased C-IMT. For example Hsue et al. reported higher C-IMT measurements in HIV-infected subjects compared to age-matched controls. Similar findings were reported by these same authors in a recent cross-sectional study, in which they also showed higher cytomegalovirus (CMV)-specific interferon-γ CD8 T-cell responses in HIV-infected compared to uninfected subjects, which were positively associated with greater C-IMT. This suggests that immune activation may be one of the additional factors contributing to atherosclerosis progression in HIV-1-infected subjects. In the current study the groups were comparable with respect to levels of CD4 and CD8 positive T-lymphocytes. Neither CD4 nor CD8 counts were significantly associated with C-IMT.

In addition, the relationship between measurements of C-IMT and other surrogate markers of atherosclerosis with regard to antiretroviral therapy, particularly protease inhibitors, have been less consistent. Whereas several studies, most of which have been cross-sectional, have reported treatment with PI to be associated with greater C-IMT or more evidence of coronary plaque formation, others have not. Of note, these studies have differed substan-

### Table 3 Results of laboratory assessments

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Protease inhibitors (n=62)</th>
<th>Non-nucleoside reverse transcriptase inhibitors (n=68)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>5.5 (4.4-6.0)</td>
<td>5.4 (4.8-6.2)</td>
<td>0.65</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
<td>1.03 (0.90-1.25)</td>
<td>1.39 (1.16-1.66)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/L</td>
<td>3.09 (2.37-3.71)</td>
<td>3.13 (2.57-3.86)</td>
<td>0.26</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>2.1 (1.4-3.4)</td>
<td>1.2 (0.9-1.7)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Apolipoprotein A, mmol/L</td>
<td>1.33 (1.16-1.45)</td>
<td>1.44 (1.30-1.59)</td>
<td>0.0008</td>
</tr>
<tr>
<td>Apolipoprotein B, mmol/L</td>
<td>1.13 (0.87-1.34)</td>
<td>1.04 (0.85-1.24)</td>
<td>0.25</td>
</tr>
<tr>
<td>AST, IU/L</td>
<td>31 (26-40)</td>
<td>29 (25-39)</td>
<td>0.18</td>
</tr>
<tr>
<td>ALT, IU/L</td>
<td>32 (22-44)</td>
<td>29 (22-42)</td>
<td>0.68</td>
</tr>
<tr>
<td>hs-CRP, mg/L</td>
<td>1.7 (0.8-4.3)</td>
<td>2.9 (1.0-5.6)</td>
<td>0.18</td>
</tr>
<tr>
<td>Glucose, mmol/L</td>
<td>4.6 (4.3-5.2)</td>
<td>5.1 (4.7-5.4)</td>
<td>0.0016</td>
</tr>
<tr>
<td>Free T4, pmol/L</td>
<td>14.0 (13.0-14.8)</td>
<td>15.1 (13.0-17.6)</td>
<td>0.35</td>
</tr>
<tr>
<td>TSH, mE/L</td>
<td>1.35 (0.96-1.90)</td>
<td>1.20 (0.98-1.80)</td>
<td>0.48</td>
</tr>
<tr>
<td>CD4+ T-cell count, cells/mm³</td>
<td>510 (350-705)</td>
<td>530 (410-680)</td>
<td>0.28</td>
</tr>
<tr>
<td>CD4+ T-cell count, %</td>
<td>25 (19-30)</td>
<td>27 (19-35)</td>
<td>0.095</td>
</tr>
<tr>
<td>CD8+ T-cell count, cells/mm³</td>
<td>890 (625-1410)</td>
<td>910 (670-1390)</td>
<td>0.80</td>
</tr>
<tr>
<td>CD8+ T-cell count, %</td>
<td>46 (40-53)</td>
<td>44 (37-54)</td>
<td>0.67</td>
</tr>
<tr>
<td>CD4/CD8 ratio</td>
<td>0.53 (0.37-0.70)</td>
<td>0.61 (0.39-0.86)</td>
<td>0.35</td>
</tr>
</tbody>
</table>

Footnote: HDL denotes high-density lipoprotein, LDL low-density lipoprotein, AST aspartate aminotransferase, ALT alanine aminotransferase, hs-CRP high sensitivity C-reactive protein, and TSH thyroid stimulating hormone.
tially with respect to the selection of patients and in particular with respect to inclusion criteria concerning prior exposure to antiretroviral therapy. To the best of our knowledge, few, if any, of these studies have required patients to have been exposed to just PI- or NNRTI-containing therapy without allowing them to have switched between these two antiretroviral drug classes. Furthermore, none of these earlier studies required patients to have plasma HIV-1 RNA levels suppressed to below 50 copies per milliliter as was the case in our study, in order to minimize the potentially confounding effect of ongoing HIV-1 replication on atherogenesis. Our finding that PI-containing combination therapy is associated with greater C-IMT than NNRTI-containing therapy is consistent with the results as obtained by Maggi et al.\textsuperscript{27} Using an ultrasound color Doppler technique, they reported a higher prevalence of pathological C-IMT (defined as > 1 mm) and/or plaques in 105 patients (53% with viral load below the limit of detection) treated for a median of 26 months with PI-based CART (52.4%) than in 125 PI-naïve patients (59% with viral load below the limit of detection) treated for a median of 24 months with NNRTI-based CART (15.2%).

### Table 4 Results of univariate and multivariate linear regression analyses with C-IMT as the dependent variable

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Univariate Estimate (mm)</th>
<th>$P$</th>
<th>Multivariate Estimate (mm)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Use of PI</td>
<td>+0.10</td>
<td>0.0003</td>
<td>+0.10</td>
<td>0.0001</td>
</tr>
<tr>
<td>Use of ritonavir (any dose)</td>
<td>+0.072</td>
<td>0.012</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Ritonavir dose (per 100 mg)</td>
<td>+0.006</td>
<td>0.37</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Duration of ART use (per year)</td>
<td>+0.018</td>
<td>0.0003</td>
<td>+0.011</td>
<td>0.017</td>
</tr>
<tr>
<td>Pack years smoking (per 10 years)</td>
<td>+0.020</td>
<td>0.04</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>LDL-cholesterol</td>
<td>+0.023</td>
<td>0.11</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>HDL-cholesterol</td>
<td>-0.074</td>
<td>0.026</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>+0.021</td>
<td>0.016</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Apolipoprotein A1</td>
<td>-0.11</td>
<td>0.046</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Apolipoprotein B</td>
<td>+0.15</td>
<td>0.0025</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>hs-CRP</td>
<td>+0.0025</td>
<td>0.18</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Glucose</td>
<td>+ 0.0015</td>
<td>0.87</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Diabetes</td>
<td>+0.040</td>
<td>0.73</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Pulse pressure (per 10 mmHg)</td>
<td>+0.016</td>
<td>0.30</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>SBP (per 10 mmHg)</td>
<td>+0.023</td>
<td>0.022</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>DBP (per 10 mmHg)</td>
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<td>0.006</td>
<td>+0.025</td>
<td>0.097</td>
</tr>
<tr>
<td>Body Mass Index</td>
<td>+0.017</td>
<td>0.0005</td>
<td>+0.011</td>
<td>0.015</td>
</tr>
<tr>
<td>Age (per 10 years)</td>
<td>+0.069</td>
<td>&lt;0.0001</td>
<td>+0.050</td>
<td>0.0003</td>
</tr>
<tr>
<td>Framingham Risk Score (per percent increase)</td>
<td>+0.008</td>
<td>&lt;0.0001</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

Footnote: ART denotes antiretroviral therapy, LDL low-density lipoprotein, HDL high-density lipoprotein, hs-CRP high sensitivity C-reactive protein, SBP systolic blood pressure, DBP diastolic blood pressure, NS = not significantly associated in the multivariate analyses, therefore not included in the final multivariate model.
Our study has several limitations in addition to its modest sample size. First, the lack of an HIV-negative control group and an HIV-positive untreated group renders it impossible to draw any conclusions from our study as to the contribution of HIV on atherogenesis. Nevertheless, as mentioned before, most studies to date strongly suggest the virus or the immune dysfunction associated with it to indeed be a relevant contributor. Second, given the cross-sectional nature of our study design, we cannot judge to which extent the use of PI- or NNRTI-containing treatments differ with respect to their effect on atherosclerosis progression. The results from a number of studies however, do provide circumstantial evidence that the rate of atherosclerosis progression may indeed be greater for patients exposed to PI. Thirdly, we have no information regarding the presence of insulin resistance in our patients, although we did record the presence or absence of diabetes mellitus and did perform fasting glucose levels. Finally, given that the number of female patients enrolled into our study was limited, we cannot determine whether our findings equally apply to both genders.

In conclusion, our results indicate that treatment of HIV-1-infected patients for two years or more with PI-based compared to NNRTI-based CART is associated with a relative C-IMT increase in the PI group. This is in accordance with the reported higher odds for CVD in patients using PI. However, we want to emphasize that no cause-effect relationship was established between PIs and C-IMT. Also, this difference was not fully explained by a more favorable impact of NNRTI-based CART on HDL-C and apo-A1 levels. These findings stress the impact of antiretroviral therapy on atherogenesis, but also suggest that traditional CVD risk factors in the HIV-infected patient population should be identified and properly managed.
Reference List


Nevirapine Increases High-Density Lipoprotein Cholesterol Concentration by Stimulation of Apolipoprotein A-I Production

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Submitted for publication
Abstract

Objective: To identify the mechanism by which the non-nucleoside reverse transcriptase inhibitor nevirapine (NVP) increases high-density lipoprotein cholesterol in treatment-experienced HIV-1 infected patients.

Methods: Fourteen HIV-1 infected patients, with stably suppressed HIV-1 viral load (plasma HIV-1-RNA <50 copies/ml) using AZT/3TC/abacavir for ≥6 months, added NVP to their current antiretroviral regimen. Patients received a primed bolus infusion of the stable isotope L-[1-13C]-valine for 12 hours before, as well as 6 and 24 weeks after the addition of NVP in order to study apolipoprotein A-I (apoA-I) kinetics. Absolute production rate (APR) and fractional catabolic rate (FCR) of apoA-I were calculated using SAAM-II modelling. Major HDLc-modulating enzymes were also assessed.

Results: plasma ApoA-I (14±4%) and HDLc (19±5%) levels increased significantly after 24 weeks of NVP treatment. Concomitantly, apoA-I production rate at 24 weeks increased by 16±6% (p=0.03), whereas apoA-I catabolism did not change. Slight increases were observed in activity of lecithin:cholesterol acyltransferase and cholesteryl ester transfer protein.

Conclusions: NVP increases apoA-I production which contributes to the marked HDLc increase following introduction of NVP-containing regimens. Changes in apoA-I and HDLc plasma levels could not be accounted for by the observed changes in HDLc-modulating enzymes. In view of the potent anti-atherogenic effects of apoA-I, the observed apoA-I increase may contribute to the favorable cardiovascular profile of NNRTIs such as NVP.
Introduction

Combination antiretroviral therapy (CART) for HIV-1 infection, comprising protease-inhibitors (PIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), and nucleoside reverse transcriptase inhibitors, has led to a dramatic reduction in HIV-related morbidity and mortality. However, most of the PIs have been shown to induce lipid changes, predominantly increases in low-density lipoprotein cholesterol (LDLc) and/or triglycerides (TG) levels which have a pro-atherogenic effect [1]. The clinical impact of these PI-induced lipid changes has recently been substantiated, as HIV infected individuals treated with PI-based CART were shown to have an increased risk of developing cardiovascular disease (CVD), in part due to changes in lipids [2]. In contrast, in the same study, NNRTI-based CART, which is characterized by an increase in high-density lipoprotein cholesterol (HDLc)[3, 4], was not found to be associated with a greater CVD risk.

HDL is a key player in the anti-atherogenic reverse cholesterol transport. In addition, HDL has several other protective properties, including anti-inflammatory, anti-oxidative and anti-coagulant effects [5]. NNRTIs have consistently been associated with an increase in HDLc, the magnitude of which varies from 20% to 49% dependent upon the characteristics of the patient population investigated [3, 4, 6-8]. Overall, the HDLc increase has been shown to be most pronounced with the use of nevirapine as compared to efavirenz [3, 9]. Since it has proven difficult to develop selective HDLc increasing compounds [10], the significant increase in HDLc following NNRTIs is clearly of interest. The mechanisms, however, by which NNRTIs mediate these changes, have not yet been unraveled. While HDLc increase can be the result of increased production of apolipoprotein (apo) A-I [11], the most common mechanism responsible for increasing HDLc levels is due to decreased HDLc catabolism via modulation of transfer proteins involved in HDL-remodeling and degradation [12].

To evaluate the mechanism by which NVP increases HDLc, we measured the in vivo kinetics of apoA-I using stable isotope infusion both before, as well as 6 and 24 weeks after adding NVP to the existing antiretroviral regimen of HIV-1 infected patients, characterized by stably suppressed plasma HIV-1-RNA levels to < 50 copies/mL for ≥ 6 months. Concomitantly, we measured plasma lipid concentrations as well as the activity of transfer proteins involved in HDL-metabolism.
Methods

Between December 2003 and September 2005, 14 male HIV-1 infected patients 18 years or older were included in this multicenter trial. Patients were recruited from the outpatient clinics of the Academic Medical Center and the Onze Lieve Vrouwe Gasthuis hospital, both located in Amsterdam, and from the HIV outpatient clinic of the University College London in the United Kingdom. Patients were included if they had been using a triple combination drug regimen of zidovudine, lamivudine and abacavir for at least 6 months prior to study entry while having an undetectable viral load, i.e. plasma HIV-1 RNA ≤50 copies/mL, during that period. Patients were excluded from the study if they met any of the following criteria: previous exposure to NNRTI, fasting hypertriglyceridemia (>5.65 mmol/L), a documented history of diabetes mellitus or hypertension, or CD4 counts > 250 cells/mm³ (women) or > 400 cells/mm³ (men). The study lasted for 24 weeks. None of the patients were taking medication known to affect plasma lipid levels. Compliance of study drug intake was verified by measuring NVP plasma levels at week 2, 6 and 24 of the study. The study protocol was approved by the institutional review boards of all 3 participating hospitals. All subjects provided written informed consent.

Experimental protocol

ApoA-I kinetic studies using stable isotopes were performed at 0 weeks (baseline), 6 weeks and 24 weeks after adding NVP to the antiretroviral regimen. After an overnight fast subjects were given a primed constant intravenous infusion of L-[1-13C]-valine. A bolus of 15 µmol per kilogram body weight of L-[1-13C]-valine was administered intravenously followed by a 12 hour constant infusion of 15 µmol/kg/h. Subjects remained fasting throughout the entire study day having free access only to drinking water. Blood samples (20 ml) were collected from an antecubital vein of the contralateral arm at regular intervals (-5 minutes, prior, and 15, 30 and 45 minutes as well as 1, 1.5, 2, 3, 4, 5, 6, 8, 10 and 12 hours after start of the isotope infusion) in tubes containing EDTA and heparin, respectively.

Plasma was separated by centrifugation at 3,500 rpm for 15 min at 4 ºC. HDL was purified by means of density gradient ultracentrifugation. ApoA-I was purified by SDS-PAGE using 12.5% gels. ApoA-I bands were excised from the polyacrylamide gels, hydrolyzed in 12 N HCl at 110 ºC for 18 hours. The tracer-to-tracee ratio of L-(1-13C)-valine was measured on an isotope ratio mass spectrometer (Delta Plus, Thermo Scientific, Bremen Germany) at the different time points. The tracer-to-tracee ratio of α-ketoisovalerate in plasma was measured on a GC-MSD system (Agilent technologies, Palo Alto, CA, USA) as described previously [13].

Kinetic Analysis

Kinetic analysis of tracer-to-tracee ratios was achieved using computer software for Simulation, Analysis and Modeling (SAAM) II (version 1.2, University of Washington, Seattle, WA). HDL apoA-I kinetics were assessed using a one-compartmental model as described previously.
The fractional synthetic rate (FSR), i.e. the proportion of apoA-I entering the pool per unit of time (pool d⁻¹), and the absolute production rate (APR), i.e. the amount of apoA-I entering the pool per unit of time (mg kg⁻¹ d⁻¹), were calculated. Absolute production rate was determined using the formula: \[ APR = (FSR) \times (\text{plasma apoA-I concentration}) \times (\text{plasma volume}) \times \frac{1000}{\text{body weight}} \]. The apoA-I pool size was calculated by multiplying plasma apoA-I by 0.045 (L kg⁻¹), assuming a plasma volume of 4.5% of body weight [17]. The apoA-I pool was considered to be constant during the experiment. If one assumes steady-state conditions [17], FSR equals the fractional catabolic rate (FCR). To estimate the apoA-I synthesis we used the plateau of \( \alpha \)-ketoisovalerate tracer-to-tracee ratio as precursor pool enrichment. ApoA-I kinetics were calculated using the following function: \[ A(t) = A_p(1 - \exp \left[ -k(t-d) \right]) \], where \( A(t) \) is the apolipoprotein enrichment at time \( t \), \( A_p \) the enrichment at the plateau of the \( \alpha \)-ketoisovalerate, \( d \) the delay between the beginning of the experiment and the appearance of tracer in the apolipoprotein and \( k \) the fractional synthetic rate of the apolipoprotein [18].

**Lipid and lipoprotein modifying proteins and enzymes**

Phospholipid transfer protein (PLTP) activity was measured in a liposome vesicles-HDL system as described previously [19]. PLTP mass was determined as published previously [20]. Lecithin:cholesterol acyltransferase (LCAT) activity was determined using excess exogenous substrate containing [\( ^{1} \text{H} \)]-cholesterol as described [19, 21]. LCAT and PLTP activities were expressed as percentage of normal human reference plasma pool, which was set at 100% (equivalent to 65 nmol/ml plasma/h for LCAT and 13.9 \( \mu \)mol/ml per h for PLTP-activity). Cholesteryl ester transfer protein (CETP) concentration was determined using two-antibody ELISA. A combination of monoclonal antibodies TP1 and TP2 was employed as coating antibodies and monoclonal antibody TP20, labeled with digoxigenine, as the secondary antibody. CETP activity was determined after removal of VLDL+LDL from each sample, as published previously [22].

**Biochemical analyses**

Total cholesterol, HDLc and triglycerides were determined with commercially available enzymatic methods (Roche Diagnostics GmbH, Mannheim, Germany). LDLc was calculated using the Friedewald formula. ApoA-I and apoB were determined by nephelometric immunochemistry (Behring, Marburg, Germany).

**Statistical analysis**

In order to detect a change in FSR exceeding 20% \( (\alpha=0.01, \beta=0.2) \), at least 10 patients had to be included in the study. All analyses were performed using the percent change from baseline in all patients who took at least one dose of NVP (modified intention to treat). Initially the FSR measurements were planned to be tested only at week 0 and week 6. Therefore, changes in the FSR and APR outcomes from week 0 to week 6 were tested by the Wilcoxon signed rank test. After inclusion of the first 2 patients, the study protocol was amended to additionally...
measure FSR and APR at week 24 given the delayed onset increase in HDLc and apoA-I. The changes over the full 24 week period were analyzed using a generalized linear model that takes repeated measurements into account (PROC MIXED in SAS). The most appropriate covariance structure was selected based on the likelihood ratio test using a restricted maximum likelihood model for estimations. In this model, missing data for a particular patient were imputed with a value based on the mean value of all available data of all patients for a given parameter, given the specified covariate structure.

If the outcome parameters were normally distributed a generalized linear model was used as described before. If they were not normally distributed, the Wilcoxon signed rank test was performed. Regarding the Wilcoxon signed rank test, missing data were treated as missing. The data in figures and tables are described as means and standard errors or as medians and interquartile ranges or numbers and percentages, as appropriate.

Table 1  Baseline Characteristics

<table>
<thead>
<tr>
<th>NVP</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients (%)</td>
<td>14 (100)</td>
</tr>
<tr>
<td>Male, n(%)</td>
<td>14 (100)</td>
</tr>
<tr>
<td>Age, years</td>
<td>44 (34-50)</td>
</tr>
<tr>
<td>Race, n(%)</td>
<td></td>
</tr>
<tr>
<td>Caucasian</td>
<td>13 (92.9)</td>
</tr>
<tr>
<td>Black</td>
<td>1 (7.1)</td>
</tr>
<tr>
<td>Height, cm</td>
<td>179 (174-184)</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>75.5 (69.0-92.0)</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>24 (22-26)</td>
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<tr>
<td>History of alcohol use, n(%)</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>2 (14.3)</td>
</tr>
<tr>
<td>Yes</td>
<td>12 (85.7)</td>
</tr>
<tr>
<td>Smoking history, n(%)</td>
<td></td>
</tr>
<tr>
<td>Never smoked</td>
<td>4 (28.6)</td>
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<tr>
<td>Ex smoker</td>
<td>7 (50.0)</td>
</tr>
<tr>
<td>Current smoker</td>
<td>3 (21.4)</td>
</tr>
<tr>
<td>Ex smokers</td>
<td></td>
</tr>
<tr>
<td>years since stopping smoking</td>
<td>38 (19-43)</td>
</tr>
<tr>
<td>HIV RNA, log10 copies/mL</td>
<td>1.69 (1.69-2.12)</td>
</tr>
<tr>
<td>CD4⁺ count, cells/m³</td>
<td>515 (200-840)</td>
</tr>
</tbody>
</table>

Data are presented as median (interquartile range) unless specified otherwise. BMI denotes body mass index.
Results

Fourteen male HIV-1 infected patients were included, 13 of whom were Caucasian and 1 was black. The baseline characteristics of the patients are shown in Table 1. The median (interquartile range) age of the patients was 44 (34-50) years. Median HIV RNA at study-entry was 1.69 (1.69-2.12) log10 copies/mL, and the median CD4+ count was 515 (200-840) cells/mm3. Nevirapine had to be discontinued in one patient who experienced transaminase elevations which exceeded 5 times the upper limit of normal within the first 2 weeks of therapy. Discontinuation of nevirapine resulted in normalization of his liver transaminases within 3 weeks. Another patient had a rash which was assessed to be caused by the use of nevirapine leading to discontinuation of drug use.

ApoA-I kinetics
ApoA-I plasma levels increased from 1.19 g/L at baseline to 1.34 g/L at 24 weeks, a mean (±SE) increase of 14 ± 4% (p=0.005). We observed a gradual increase in mean apoA-I pool size between 6 and 24 weeks of therapy with NVP. When analyzing each patient separately all patients except one showed increases in apoA-I pool size at 24 weeks of NVP therapy (data not shown). ApoA-I FCR did not change significantly throughout the study (Figure 1). The median (interquartile range) APR of apoA-I increased from 9.88 (8.20 to 10.59) mg/kg/day at baseline to 10.05 (8.78 to 11.23) mg/kg/day at 24 weeks of NVP treatment (16±6% increase; p=0.03). The absolute increase (SE) of apoA-I APR of 1.36 mg/kg/day was also significant using a mixed models analysis (p=0.02). At week 6 no significant change was seen in APR. We performed an exploratory post-hoc analysis comparing the changes between week 6 and week 24 showing an increase (interquartile range) of 22% (10% - 31%) using the wilcoxon signed rank test (p=0.0273).

| Table 2 Lipid changes after 6 and 24 weeks of nevirapine treatment |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                | Baseline        | Week 6          | Week 24         | % change bl to wk 6 | p-value week 6 | % change bl to wk 24 | p-value week 24 |
| **TC, mmol/L** | 4.16 (3.78-4.53) | 4.42 (4.24-4.84) | 4.42 (4.01-5.46) | 6 (4) | 0.163 | 11 (4) | 0.012 |
| **HDLc, mmol/L** | 1.13 (0.95-1.37) | 1.15 (1.06-1.34) | 1.38 (0.98-1.46) | 4 (4) | 0.334 | 19 (5) | 0.003 |
| **LDLc, mmol/L** | 2.41 (2.07-2.80) | 2.68 (2.34-2.93) | 2.86 (2.19-3.36) | 5 (5) | 0.370 | 13 (5) | 0.034 |
| **TG, mmol/L**  | 1.35 (0.87-1.74) | 1.55 (1.14-2.06) | 1.04 (0.91-1.32) | 53 (36) | 0.168 | -6 (11) | 0.583 |
| **ApoA-I, g/L** | 1.19 (1.10-1.35) | 1.17 (1.07-1.39) | 1.34 (1.13-1.57) | -4 (7) | 0.570 | 14 (4) | 0.005 |
| **ApoB, g/L**   | 0.83 (0.77-0.98) | 0.91 (0.75-0.98) | 0.94 (0.81-1.07) | -7 (7) | 0.323 | 9 (4) | 0.074 |

Data are presented as median (interquartile range). TC denotes total cholesterol, HDLc high-density lipoprotein cholesterol, LDLc low-density lipoprotein cholesterol, TG triglycerides, ApoA-I apolipoprotein A-I, and ApoB apolipoprotein B, % change bl to wk 6 denotes percent change from baseline to week 6 of nevirapine treatment, % change bl to wk 24 denotes percent change from baseline to week 24 of nevirapine treatment.

To convert cholesterol values from mmol/L to mg/dL multiply by 38.67. To convert triglycerides values from mmol/L to mg/dL multiply by 88.57. Lipid parameters were analyzed using an intention-to-treat analysis (n=14) in which missing values were imputed with a value based on the mean value of all available data of all patients for a given parameter.
**Lipids**

Baseline median (interquartile range) plasma total cholesterol, HDLc, LDLc and TG were 4.16 (3.78-4.53), 1.13 (0.95-1.37), 2.41 (2.07-2.80) mmol/L and 1.35 (0.87-1.74) mmol/L respectively. After 6 weeks of NVP treatment, no significant changes were observed in plasma levels of apoA-I or HDLc. At 24 weeks, however, HDLc levels had increased significantly by 19±5% to 1.38 (0.98-1.46) mmol/L (p=0.003) while LDLc increased by 13±5% to 2.86 (2.19-3.36) mmol/L. As a consequence, total cholesterol increased with 11±4%, while triglyceride levels remained unaffected (Table 2).

**Lipid and lipoprotein modifying proteins and enzymes**

Table 3 shows data on the effect of nevirapine treatment on lipid and lipoprotein modifying proteins and enzymes. CETP activity and LCAT activity increased significantly at 24 weeks. PLTP activity was unaffected during the entire treatment period (Table 3).

![Figure 1](image-url)  
Percent change in apolipoprotein A-I absolute production rate and fractional catabolic rate after 6 and 24 weeks of nevirapine treatment

Data are presented as mean percent change. Bars represent standard errors.

* not significant

# p=0.03
Discussion

To our knowledge the present study is the first to show that in patients receiving NVP, the increase in HDLc and its major apolipoprotein, apoA-I, results from a stimulation of the apoA-I production rate. In contrast, apoA-I catabolism remained largely unaffected. In view of the potent anti-atherogenic effect of apoA-I, this effect is likely to contribute to the lack of adverse cardiovascular effects of NNRTI as opposed to PI-containing CART regimens in HIV-1 infected subjects.

ApoA-I kinetics

The observed absolute production rates of apoA-I at baseline are comparable with the production rates reported in healthy individuals [18, 23, 24], subjects with metabolic syndrome [25], and CETP-inhibitor treated subjects [26, 27]. After 24 weeks of NVP treatment, apoA-I APR had increased significantly by 16%. Although the latter may seem modest, it compares favorably to changes reported for other drugs. Peroxisome proliferator-activated receptor alpha (PPARα) stimulation has been reported to increase APR by approximately +10% [28]. In this respect, the effect of NVP clearly exceeds that of fibrates, which have been designed as lipid modulating drugs targeting low HDLc and high TG levels[29]. The fractional catabolic rate of apoA-I remained unchanged. Of note, a constant fractional clearance combined with an increased pool size indicates that the absolute clearance rate of apoA-I is also increased. Since a primary increase in absolute clearance, however, cannot account for an increased apoA-I plasma concentration, the change in apoA-I clearance likely reflects a secondary increase following increased apoA-I production.

The mechanism of action leading to enhanced apoA-I production cannot be directly addressed by the present study. In comparison, several PPARα agonists are being developed which mainly increase production of ApoA-I as well as of ApoA-II [30] [31], but also decrease plasma TG levels by decreasing apoCIII and lipoprotein lipase expression. However in the present study no obvious changes in TG metabolism have been observed which is not in favor for a PPAR alpha mediated process. Alternatively, apoA-I can be increased by stimulating the expression of the nuclear receptor, liver X receptor (LXR). These LXR agonists, however, also strongly affect hepatic TG metabolism, as a direct consequence of fatty acid synthase stimulation [32], resulting in a strong increase in hepatic secretion of TG-rich lipoproteins. In view of the absence of any effect on triglycerides, a major role for LXR activation also does not seem to be a likely explanation for the present results. Finally, 1-hydroxyalkyl-3-phenylthioureas, compounds based on benzodiazepines, have also been shown to increase apoA–I production through a non-PPAR/ non-LXR mechanism [33, 34]. The mechanism of action for these compounds however has not been elucidated. Clearly, further research is needed to identify the exact molecular mechanism contributing to the increased apoA-I production rates when various drugs are used.
Table 3  Percent change in activity and/or mass of HDL-modifying enzymes after 24 weeks of nevirapine treatment

<table>
<thead>
<tr>
<th>Parameter</th>
<th>% change from baseline at week 24</th>
<th>p-value week 24</th>
</tr>
</thead>
<tbody>
<tr>
<td>LCATa</td>
<td>9 (3)</td>
<td>0.02</td>
</tr>
<tr>
<td>CETPm</td>
<td>10 (8)</td>
<td>NS</td>
</tr>
<tr>
<td>CETPa</td>
<td>14 (4)</td>
<td>0.003</td>
</tr>
<tr>
<td>PLTPm</td>
<td>-7 (4)</td>
<td>NS</td>
</tr>
<tr>
<td>PLTPa</td>
<td>4 (2)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Data are presented as mean (±SE). LCATa denotes lecithin:cholesterol cyctransferase activity, NS not significant, CETPm cholesteryl ester transfer protein mass, CETPa cholesteryl ester transfer protein activity, PLTPm phospholipid transfer protein mass, PLTPa phospholipid transfer protein activity

**NVP and HDL increase**

We previously observed that changes in HDLc reached steady state approximately 24 weeks following NVP initiation [4, 35]. In the present study, a 19% increase in HDLc levels and a 14% increase in plasma apoA-I levels were observed 24 weeks after NVP initiation. These findings are in accordance with previous studies in CART-experienced patients in whom replacement of PIs with NVP was associated with a 20% HDLc increase [6, 8]. In contrast, early and larger increases in HDLc up to 49% have been reported following the initiation of NVP in antiretroviral naïve subjects [3]. Since HDL is an inverse acute phase reactant, a substantial part of the increase in treatment naïve patients has been attributed to a ‘return-to-health’ phenomenon following effective first time suppression of HIV-1 infection [36]. Interestingly, the observation that 6 weeks of NVP had not yet significantly affected HDLc levels suggests the absence of a ‘return-to-health’ phenomenon in the present study. The latter is in line with the fact that we only included patients with durably suppressed HIV-1 infection. However, it should be noted that the apparent decrease in APR at week 6 was predominantly caused by a single individual, showing an unexplained profound decrease. Exclusion of this subject resulted in a more stable pattern of a progressive increase in APR. Conversely, the onset of HDLc increase between 6-24 weeks following NVP initiation implies a delayed mechanism of action. However, since the exact mechanism by which NVP elicits HDLc increase cannot be addressed in the present in vivo study, further experimental studies are required to elucidate the causes for the late onset.

**Lipid and lipoprotein modifying proteins and enzymes**

Changes in HDLc levels can be a result of changes in the activity of lipid and lipoprotein modifying proteins and enzymes [12]. We measured several principal parameters that are known to affect changes in HDL metabolism. At 24 weeks, we observed a modest increase in both LCAT and CETP-activity. While increased LCAT activity can be expected to increase HDLc, an increased CETP activity causes a decrease in HDLc. These minor changes should, however, not be used to delineate causal relations with regard to systemic HDLc concentrations in the patients studied.
Clinical implications

The importance for cardiovascular prevention in HIV-infected patients has been widely acknowledged. Recent data from the DAD study support the need to appropriately manage traditional cardiovascular risk factors in HIV, and to refrain from using PI-based CART regimens in patients at increased CVD risk whenever this is possible without jeopardizing appropriate and sustained control of HIV replication [2]. The DAD study also suggested that NNRTI-based regimens may be an appropriate alternative in that context. The SMART study, which looked at the effect of treatment interruption on patient’s health, supported this notion in a recent presentation of their data [37]. It was demonstrated that patients on NNRTI therapy (n=1980) had a hazard ratio (treatment interruption arm/continuous therapy arm) for CVD events of 2.07 (0.89-4.84). The risk appeared to be exclusively related to patients in the drug conservation arm. In further analysis the SMART study also showed that patients in the treatment interruption arm suffered the greatest decline in HDL, especially if they had been on NNRTI therapy at the time they entered the study. This leads to the speculation that the mechanism of increased risk for CVD in patients taking NNRTIs in the SMART study is the precipitous decline in HDL which occurs when these drugs are stopped.

Study limitations

In the SMART study increased levels of IL-6 and amyloid P in the drug conservation group were associated with increased incidence of CVD [38] Whether these pro-inflammatory changes are specifically inhibited by NVP and may also contribute to cardiovascular protection should be investigated in future studies. In the present study, we only evaluated the effect of NVP on apoA-I kinetics. As such, it remains to be established whether other NNRTIs, such as EFV, exert similar effects.

NVP also increase pro-atherogenic lipid fractions. However, the numerically greater increase in HDL and apoAI (19% and 14%, respectively), as compared to LDL and apoB (13% and 9% respectively) can be expected to provide an overall benefit on cardiovascular risk, which has been substantiated in the DAD study [2]. In line, a net anti-atherogenic effect of NVP is supported by recent findings from our group, in which NVP was associated with less thickening of carotid intima-media thickness, an established surrogate marker for CVD, when compared with the use of protease inhibitors in HIV-1 infected patients [39]

Conclusion

The present study shows a clear increase in plasma levels of HDLc and apoA-I following the administration of nevirapine in HIV-1 infected patients with durable virus suppression during prior treatment with a combination of zidovudine, lamivudine and abacavir. These effects were shown to result from an increased production of apoA-I. The finding of a stimulation of apoA-I production by nevirapine may assist ongoing efforts aimed at unraveling novel therapeutics to increase apoA-I/HDLc levels in patients at increased cardiovascular risk in general.
Acknowledgements

We would like to thank our trial nurses Hans-Erik Nobel, Lucy Schrijnders and Dee Mullan for their administrative, technical and laboratory support. Joram van Miert is greatly acknowledged for his assistance with the kinetic studies. We thank Geesje Dallinga and Jan Albert Kuivenhoven for their excellent advice regarding laboratory methodology and CETP measurements. We are greatly indebted to Matti Jauhiainen for the PLTP measurements. We thank Dr Ian Williams for recruiting the UK participants. This study was financially supported by Boehringer Ingelheim Pharmaceuticals.
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Pharmacokinetics and Pharmacodynamics of Combined Use of Lopinavir/ritonavir and Rosuvastatin in HIV-infected Patients

M.J. van der Lee
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Abstract

Background: Lopinavir/ritonavir-containing antiretroviral therapy can cause hyperlipidaemia in HIV-infected patients. However, most statins are contraindicated due to drug-drug interactions. Rosuvastatin undergoes minimal metabolism by CYP 450, so no CYP 450-based interaction with lopinavir/ritonavir is expected. This pilot study explored the lipid-lowering effect of rosuvastatin and assessed the effect of lopinavir/ritonavir on the pharmacokinetics of rosuvastatin and vice versa.

Methods: HIV-infected patients on lopinavir/ritonavir (viral load<400 copies/mL) with total cholesterol (TC)>6.2mmol/L were treated with rosuvastatin for 12 weeks, starting on 10 mg QD. If fasting target values (TC<5.0mmol/L; HDL-c>1.0mmol/L; LDL-c<2.6mmol/L; TG<2.0mmol/L) were not reached, rosuvastatin was escalated to 20mg or 40mg at week 4 and 8. Plasma lopinavir/ritonavir trough levels (Cmin) were drawn at week 0, 4, 8, 12; rosuvastatin Cmin at week 4, 8, 12.

Results: 22 patients completed the study. Mean reductions in TC and LDL-c from baseline to week 4 (on rosuvastatin 10mg QD) were 27.6% and 31.8%. Lopinavir/ritonavir concentrations were not influenced by rosuvastatin (p=0.44 and 0.26, repeated-measures analysis). Median (IQR) rosuvastatin Cmin for 10mg, 20mg and 40mg QD were 0.97 (0.70–1.5), 2.5 (1.3–3.3) and 5.5 (3.3–8.8) ng/mL. Rosuvastatin was well tolerated; three patients experienced transient muscle pain.

Conclusions: Rosuvastatin appeared to be an effective statin in hyperlipidaemic HIV-infected patients. Lopinavir/ritonavir levels were not affected by rosuvastatin, whilst rosuvastatin levels unexpectedly appeared to be increased 1.6-fold as compared to data from healthy volunteers. So, probably another mechanism other than CYP 450 is involved in this interaction. Until safety and efficacy have been confirmed in larger studies, the combination of rosuvastatin and lopinavir/ritonavir should be used with caution.
Introduction

Lopinavir/ritonavir is one of the most widely-used HIV-protease inhibitors (PIs). One of the drawbacks when using lopinavir/ritonavir is the frequent development of hyperlipidaemia [1]. A strategy to manage lopinavir/ritonavir-induced hyperlipidaemia may be treatment with lipid-lowering drugs such as 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) and/or fibrates [2;3]. The concomitant use of such drugs with PIs has been hampered by the occurrence of drug-drug interactions [4]. Most statins are metabolized by cytochrome P450 (CYP450), while PIs, including lopinavir/ritonavir, are strong inhibitors of this enzyme.

Hence, there is a clear need for statins which have both potent lipid-lowering effects and are not subject to such drug-drug interactions. The newly introduced statin rosuvastatin may fulfil these requirements. CYP450-based metabolism does not play an important role in the clearance of rosuvastatin [5;6]. Rosuvastatin is only minimally metabolized by CYP3A4 [7]. Furthermore, rosuvastatin has been demonstrated to be an effective statin in HIV-uninfected patients [8]. However, the pharmacokinetics of rosuvastatin when used concomitantly with PIs has not yet been investigated.

The primary objective of this pilot study was to explore the effect of rosuvastatin on plasma lipids in HIV-infected patients with hyperlipidaemia who were on stable lopinavir/ritonavir containing antiretroviral therapy (ART). The bidirectional pharmacokinetics of rosuvastatin and lopinavir/ritonavir and the safety of the combination were also assessed.

Methods

This pilot study was conducted from May 2004 until July 2005 at Radboud University Nijmegen Medical Centre, Academic Medical Center Amsterdam, and University Medical Centre Leiden, all in the Netherlands, and at University of Bonn and Cologne in Germany.

Study population
This study was conducted in HIV-1-infected dyslipidaemic patients, aged 18 to 65 years, stable on lopinavir/ritonavir 400/100mg BID-containing ART for at least 3 months and had signed informed consent. Hyperlipidaemia was defined as fasting total cholesterol >6.2mmol/L (239 mg/dL). Plasma HIV-1 RNA had to be below 400 copies/mL. The main exclusion criteria were: sensitivity/idiosyncrasy to rosuvastatin or chemically related compounds, a relevant history or current condition that might interfere with pharmacokinetics and pregnant or breast-feeding females. Japanese/Chinese patients were excluded because Asian patients are more likely to experience side effects of rosuvastatin such as rhabdomyolysis [9]. Other exclusion criteria
were: creatinine clearance <60ml/min (calculated from serum creatinine), fasting triglyceride level >8.0mmol/L (700mg/dL), abnormal creatine kinase levels (>10 times upper limit of normal), history of statin-related rhabdomyolysis or family history of inheritable muscle disease. The use of any statin or fibrate in the 6 weeks prior to the first dose, previous use of rosvastatin and concomitant medication, known to interfere with the pharmacokinetics of rosvastatin or lopinavir/ritonavir, were not allowed. Subjects were advised not to change their diet during the study. At screening (within 3 weeks prior to the first dose) patients’ eligibility for inclusion was established.

**Study design**
All subjects started with an oral dose of 10mg rosvastatin once-daily (QD) in addition to their regular dose of lopinavir/ritonavir (400/100mg twice-daily [BID] as soft gelatine capsules) and other components of their current ART-regimen. Patients used this combination until week 4 at which time fasting lipids were determined. The dose of rosvastatin was escalated to 20mg QD for the following 4 weeks if patients had not reached each of the four following targets derived from the Guidelines of the HIV Medical Association of the Infectious Disease Society of America (IDSA) and the Adult AIDS Clinical Trials Group [10]: total cholesterol <5.0mmol/L (192mg/dL); LDL-cholesterol <2.6mmol/L (100mg/dL); HDL-cholesterol >1.0mmol/L (40mg/dL); triglycerides <2.0mmol/L (175mg/dL). If after an additional 4 weeks of 20mg of rosvastatin QD, the above-mentioned targets were still not reached at week 8, rosvastatin was escalated once more to 40mg QD for the subsequent four weeks (up to week 12, end of study). If patients had reached all targets at week 4 or 8, they continued their current dose of rosvastatin for the remainder of the study. Patients continued using lopinavir/ritonavir 400/100mg BID during the whole study period.

Relatively short periods, i.e. 4 weeks, were chosen after which the dose of rosvastatin could be escalated if the abovementioned targets were not reached, because the maximum lipid-lowering effect of rosvastatin has mostly been shown to be achieved after a 4-week dosing period [9], and because we wanted to limit the overall duration of the trial.

The study was approved by the local ethics committees of each of the participating sites.

**Biochemistry, safety assessments and pharmacokinetic sampling**
Lopinavir/ritonavir trough levels, drawn just before the next dose (within 9–15 hours after intake), were determined at week 0, 4, 8, and 12. Rosuvastatin trough levels, 24 hours after intake, at week 4, 8, and 12. Serum biochemistry, including total cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides, hepatic and muscular enzymes were performed at week 0, 4, 8, and 12 at the local sites. Adverse events were assessed at each of these time points.

Total cholesterol, HDL-cholesterol and triglycerides were measured using enzymatic colorimetric tests. In Nijmegen, Bonn, Cologne, and Amsterdam LDL-cholesterol was calculated from the Friedewald formula [11]. In Leiden LDL-cholesterol was directly measured with a
colorimetric test; In Cologne this assay was used if TG>4.5mmol/L. If in Bonn patients had TG>4.5mmol/L, LDL-cholesterol could not be calculated; In Nijmegen and Amsterdam none of the patients experienced high TG levels.

**Bioanalysis of lopinavir/ritonavir and rosuvastatin concentrations in plasma**
Plasma samples of lopinavir/ritonavir were analyzed at the Department of Clinical Pharmacy, Radboud University Nijmegen Medical Centre. This department has established an HPLC assay for lopinavir/ritonavir, derived from an HPLC method which has been published previously [12]. Rosuvastatin plasma samples were measured at Covance Laboratories, Inc. Madison, Wisconsin, United States. The quantification of rosuvastatin in plasma was performed by automated solid-phase extraction using tandem mass spectrometric detection, as published previously [13].

**Sample size and statistical analysis**
No formal sample size calculation was performed as this was a descriptive pilot study. The changes in lipids were assessed using a Paired Samples T-test, because these parameters were normally distributed. For the comparison of lopinavir/ritonavir concentrations General Linear Model Repeated Measures analyses were performed using the logarithmic transformed trough concentrations. Statistical evaluations were carried out using SPSS® for Windows, version 12.0.1 [SPSS Inc, Chicago, IL, USA].

**Results**

**Baseline characteristics**
Twenty-two HIV-1-infected patients with undetectable viral load and without hepatitis coinfection (20 males) were included. Median age was 48 (IQR: 40-56) years. Median CD4 at baseline was 399 (IQR: 265-482) *10^6/L. Viral load and CD4 count did not change during the trial between baseline and week 12 (p=0.794 and p=0.783, respectively; Paired Samples T-test).

**Pharmacodynamics**
At baseline, all patients started using 10mg of rosuvastatin QD. At week 4, three patients continued using 10mg QD, but nineteen patients were dose escalated to 20mg of rosuvastatin QD. From week 8–12, one patient was still using 10mg; seven had been escalated to 20mg QD; fourteen to 40mg QD. One patient had developed an elevated creatinekinase level at week 4 (493 U/L, which is about 4-fold higher than the upper limit of normal) and was therefore not dose escalated although predefined lipid targets were not met.
Table 1 shows the effect of rosuvastatin on fasted total cholesterol, LDL-cholesterol, HDL-cholesterol and triglyceride levels. Triglyceride levels of three patients were not included in the data analysis. In spite of the fact that their triglyceride levels at screening were >8.0mmol/L, these patients met all other inclusion criteria and were nevertheless included to investigate the effect of rosuvastatin on total cholesterol, LDL-cholesterol and HDL-cholesterol, because the local investigator was determined to start lipid-lowering statin-therapy in them anyway.

Mean percent reduction (standard deviation [sd]) in total cholesterol, LDL-cholesterol, and triglycerides resulting from 10mg of rosuvastatin QD from week 0 to 4 was 27.6% (7.6%), 31.8% (16.7%), and 20.7% (38.5%), respectively (p<0.001, p<0.001, and p=0.022 vs. baseline; Paired Samples T-test). Mean increase in HDL-cholesterol between week 0 and 4 was 3.1% and not statistically significant (sd: 12.5%; p=0.460). The overall mean reduction in lipid levels (sd), comparing week 12 to baseline, combining data from all dose groups, was 33.8% (9.7%), 38.9% (25.8%) and 37.0% (26.1%) for total cholesterol, LDL-cholesterol and triglycerides, respectively (p<0.001, p<0.001, and p=0.001). There was a mean increase in HDL-cholesterol of 16.9% which did not reach statistical significance (sd: 39.5%; p=0.080 vs. baseline).

At week 12, 32% (7/22) of the patients met all lipid targets; when looking at each lipid parameter individually, 68% (15/22) of the patients reached the target for total cholesterol, 68% (13/19) for LDL-cholesterol, 68% (15/22) for HDL-cholesterol and 53% (10/19) for triglycerides.

Table 1 Mean total cholesterol, LDL-cholesterol, HDL-cholesterol, and triglycerides levels at week 0, 4, 8, and 12

<table>
<thead>
<tr>
<th></th>
<th>Total cholesterol Mean (sd) (mmol/L) N=22</th>
<th>LDL-cholesterol Mean (sd) (mmol/L) N=20</th>
<th>HDL-cholesterol Mean (sd) (mmol/L) N=22</th>
<th>Triglycerides Mean (sd) (mmol/L) N=19</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 0</td>
<td>7.1 (0.95)a</td>
<td>4.2 (0.99)c</td>
<td>1.2 (0.43)b</td>
<td>3.6 (1.8)c</td>
</tr>
<tr>
<td>Week 4</td>
<td>5.2 (0.81)b</td>
<td>2.8 (0.61)a</td>
<td>1.2 (0.41)b</td>
<td>2.6 (1.3)b</td>
</tr>
<tr>
<td>Week 8</td>
<td>4.9 (1.2)</td>
<td>2.7 (0.77)</td>
<td>1.2 (0.51)</td>
<td>2.2 (1.0)</td>
</tr>
<tr>
<td>Week 12</td>
<td>4.7 (0.79)</td>
<td>2.5 (0.61)d</td>
<td>1.4 (0.81)</td>
<td>2.3 (1.4)</td>
</tr>
</tbody>
</table>

*Two patients were excluded for data-analysis because of non-fasting; *One patient was excluded because of non-fasting; *Two patients were excluded because of non-fasting and one level could not be determined because of high triglyceride levels; *One level could not be determined because of high triglyceride levels

Pharmacokinetics

Lopinavir levels of twenty patients were included in our data analysis; levels of two patients were excluded, because these samples were not drawn within the prespecified time window (9–15 hours after intake). Median (IQR) lopinavir $C_{\text{min}}$ was 5.2 (3.7–6.5), 5.4 (4.1–7.6), 5.6 (4.2–7.8) and 5.2 (3.6–6.3) mg/L at week 0, 4, 8 and 12, respectively. A repeated-measures analysis showed no difference between logarithmic transformed lopinavir $C_{\text{min}}$ at weeks 0, 4, 8, and 12 (p=0.44).
For the analysis of the ritonavir trough levels, the same samples as for lopinavir data analysis were used. Median (IQR) ritonavir $C_{\text{min}}$ (9–15 hours after intake) was 0.22 (0.17–0.28), 0.25 (0.14–0.36), 0.26 (0.14–0.33) and 0.20 (0.17–0.34) mg/L at weeks 0, 4, 8 and 12, respectively (repeated-measures analysis: p=0.26).

For rosuvastatin, trough levels were available for 12, 13, and 9 patients on 10 mg, 20 mg, and 40 mg of rosuvastatin QD, respectively. Median trough levels were not provided for all patients, because in some cases, blood samples were drawn about 12 hours instead of 24 hours after intake. For nine patients rosuvastatin plasma levels were available for doses of 10mg, 20mg, as well as 40mg QD. Table 2 shows the median trough levels for the different dosages compared to those obtained from historical HIV-uninfected controls without hyperlipidaemia (measured with the same method and at the same laboratories as the samples from our trial) (data on file AstraZeneca; [14-16]). Rosuvastatin plasma trough levels appeared to be 1.6-fold higher compared to those in these historical healthy controls.

**Adverse events and safety assessments**

None of the included patients dropped out or temporarily stopped taking rosuvastatin during this 12–week trial. The reported adverse events were mild: diarrhea (N=2), headache (N=2), and a cold (N=2). Three patients experienced transient muscle pain/cramps: one on 10mg of rosuvastatin QD during week 3, one on 20mg QD during week 8–12, and one on 40mg QD during week 8–12, respectively. The second patient also had an elevated creatinekinase level of 436 U/L at week 12; rosuvastatin was stopped after the trial. After stopping, the symptoms disappeared and the creatinekinase level returned to normal. The other two patients had normal creatinekinase levels (range: 64–100 U/L) and continued to use rosuvastatin.

In addition, three patients had clinically asymptomatic elevations of creatinekinase above 250 U/L, ranging from 363 to 676 U/L. One of these patients already had an elevated level at baseline.

Median liver enzymes, comparing baseline to week 12, were as follows: median (IQR) ALAT (N=20; in two patients ALAT was not determined) and ASAT (N=22) were 25.5 (16.0-37.8) and 28.0 (20.0-35.3) at week 0 and 26.5 (22.0-47.5) and 27.5 (21.0-35.0) at week 12, respectively.
Table 2 Median rosuvastatin trough levels for 10, 20 and 40 mg compared to historical healthy controls

<table>
<thead>
<tr>
<th>Rosuvastatin dosage</th>
<th>Rosuvastatin C_min (ng/mL)</th>
<th>Ratio C_min rosuvastatin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Our trial</td>
<td>Historical healthy</td>
</tr>
<tr>
<td></td>
<td>Median (IQR)</td>
<td>controls* [14]</td>
</tr>
<tr>
<td>10 mg (N=12)</td>
<td>1.0 (0.69–1.5)</td>
<td>0.63 (0.27–1.2)</td>
</tr>
<tr>
<td>20 mg (N=14)</td>
<td>2.5 (1.3–3.3)</td>
<td>1.6 (0.54–4.1)</td>
</tr>
<tr>
<td>40 mg (N=9)</td>
<td>4.5 (3.3–7.5)</td>
<td>2.9 (1.7–3.6)</td>
</tr>
</tbody>
</table>

* Data on file AstraZeneca

Discussion

In a recently published study, performed in hyperlipidaemic HIV-1-infected patients on various ART-regimens using 10mg of rosuvastatin QD, similar reductions in lipid levels were observed following 24 weeks of treatment [17]. In HIV-negative patients with hyperlipidaemia, rosuvastatin in a dose range of 10-40mg, reduced LDL-cholesterol by 43%-63% (reviewed by Cheng [18]). For 10mg of rosuvastatin, we found in our trial, with a relatively small sample size, a somewhat lower effect on LDL-cholesterol. We cannot compare the results at week 8 and 12 of our study with other studies in HIV-negative patients because our trial was designed to titrate the patient to our predefined goals and only the poor responders were eligible for dose escalation.

The somewhat lower effect of rosuvastatin we found in our trial despite the increased rosuvastatin levels, could be explained by the fact that when plasma levels are increased, the intake of rosuvastatin in the hepatocytes, where it conducts its effect, is decreased. So, a decreased concentration of rosuvastatin in the hepatocytes could cause a decreased lipid-lowering effect.

Previous studies reported similar lopinavir trough concentrations [19;20], as we found. The same was true for ritonavir trough levels [21;22]. Our observations are in accordance with in vitro observations indicating an absence of inhibitory or inducing effects of rosuvastatin on CYP450 enzymes [7;23].

Only a few trials investigated rosuvastatin pharmacokinetics in patients with hyperlipidaemia. In contrast, pharmacokinetic studies have been performed extensively in healthy volunteers, but trough levels have only been reported in a minority of these studies [14-16]. For comparison, we used data from a study in healthy subjects on file at AstraZeneca, the manufacturer of rosuvastatin. In our trial, rosuvastatin trough levels were 1.6-fold higher as compared to the levels found in that study in healthy, HIV-uninfected subjects without hyperlipidaemia for all dosages of rosuvastatin. These higher rosuvastatin levels could not have been caused by...
a CYP\textsubscript{450}-based interaction since 90% of rosuvastatin is excreted unchanged in faeces [9] and CYP-based metabolism was anticipated not to play an important role in the clearance of rosuvastatin [7]. Furthermore, studies in healthy volunteers showed no relevant drug-drug interactions with rosuvastatin involving CYP2C9 [24], CYP2C19 [24], and CYP3A4 [25-27].

An alternative explanation for our observation might be that lopinavir/ritonavir affects a membrane transporter of rosuvastatin. In a study by Simonson [28] a significant increase in rosuvastatin exposure was observed in heart transplant recipients using ciclosporine. The mechanism was believed to be ciclosporine-mediated inhibition of the human liver transporter organic anion transporting polypeptide C (also known as OATP-1B1). OATP-C (1B1) is a transporter protein [29], likely to be involved in the hepatic uptake of rosuvastatin [30-32]. To our knowledge, no studies have been performed investigating the effect of lopinavir/ritonavir on OATP. However, ritonavir, saquinavir and indinavir are known to inhibit OATP1B1 [31;32].

A recently performed pharmacokinetic drug-drug interaction study with lopinavir/ritonavir in 15 healthy volunteers also showed an increase in rosuvastatin exposure: 2.1-fold increase in AUC and 4.7-fold increase in C\textsubscript{max} [33]. This effect on rosuvastatin AUC and C\textsubscript{max} is in agreement with the results of our trial. However, we cannot explain why Hoody did not find an effect on rosuvastatin C\textsubscript{min} while observing an increased AUC and C\textsubscript{max}.

Whether the increased rosuvastatin exposure, observed in our trial and by Hoody [33], is clinically relevant, is questionable while the orders of magnitude are less than what has been reported in previous interaction studies between several different PIs and statins other than rosuvastatin: the combination of PIs with atorvastatin (20mg QD) and simvastatin (40mg QD and 20mg QD) resulted in an increase in statin AUC by 590% [34], 3059% [35] and 505% [36], respectively.

A favourable tolerability profile of rosuvastatin including the absence of any cases of rhabdomyolysis was observed both in our study and by Calza [17]. One needs to realize however that rhabdomyolysis is a relatively rare complication of statin use, thus larger studies are needed to confirm safety of using rosuvastatin in HIV-infected patients on ART.

Our study has a number of limitations. Because of the relatively small sample size, no centralized plasma lipid measurements and the strict lipid target criteria, the results regarding safety and pharmacodynamic effects on lipids should be interpreted with caution. In addition, although patients were instructed not to change their diet during the study, formal data on diet were not collected during the trial and changes in dietary pattern may have influenced the results. Another limitation is that we did not include a randomized control group. Rosuvastatin trough levels were compared to those reported in healthy uninfected historical controls, and lopinavir/ritonavir plasma levels for each patient were compared to baseline before adding rosuvastatin. Future drug-drug interaction studies between rosuvastatin and PIs may consider including a randomized control group of HIV-infected patients using rosuvastatin in the ab-
sence of ART-regimens. Finally, in this pilot study we have chosen to only determine rosuvastatin trough levels in order not to burden patients with recording 24-hour pharmacokinetic curves. Nonetheless, determination of rosuvastatin AUCs would provide more information concerning any interaction between rosuvastatin and lopinavir/ritonavir, and for this reason we do suggest that in future trials pharmacokinetic curves are obtained.

In conclusion, in our pilot study rosuvastatin appeared to be an effective statin in hyperlipidaemic HIV-1-infected patients treated with lopinavir/ritonavir. Although plasma lopinavir/ritonavir levels were not affected by the concomitant use of rosuvastatin, rosuvastatin levels were 1.6-fold higher compared to those in HIV-uninfected healthy volunteers without hyperlipidaemia. Our findings support further research, especially a placebo-controlled randomized trial, in larger numbers of patients to more fully elucidate pharmacokinetics, efficacy, and safety of the combined use of rosuvastatin and lopinavir/ritonavir in HIV-infected patients. Until such time, the combination of rosuvastatin and lopinavir/ritonavir should continue to be used with caution.

Acknowledgements

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Summary

Atherosclerosis is a chronic disease of the arteries, which occurs at the level of the vessel wall. Cholesterol leakage in the vessel wall results in inflammatory activation of endothelial cells (endothelial dysfunction), resulting in increased expression of adhesion molecules (especially VCAM-1) on endothelial cells, which is followed by mobilization of monocytes to the site of endothelial dysfunction. The monocytes transmigrate to the subendothelial space where they acquire the properties of tissue macrophages. Transformation of these macrophages to foam cells occurs by inclusion of subendothelially located oxidized lipid particles. The local vessel wall has now become a site of inflammation with macrophages and T-lymphocytes as key players, which are a continuous source of chemo-attractants and pro-inflammatory cytokines. The subsequent gradual increase of inflammatory cells, debris and cholesterol leads to a progressive narrowing of a blood vessel, the atherosclerotic plaque. Proliferating smooth muscle cells in the plaque, producing abundant extracellular matrix, may render the plaque stable. However, the fibrous capsule covering the plaque can also rupture under the influence of matrix metallo proteinases, produced by the activated macrophages. Rupture of the plaque is associated with the release of tissue factor and leads to acute coagulation of the blood. The ensuing thrombus formation may occlude the blood vessel. Prolonged occlusion of the blood vessel may lead to an acute myocardial infarction (when the coronary arteries are involved) or cerebral infarction (when the cerebral arteries are involved). Reduction in LDL-C through drug treatment with statins may further inhibit growth of a plaque and very aggressive reduction of LDL-C can sometimes even lead to regression of atherosclerotic plaques. Statins have also shown their value in primary prevention for cardiovascular disease.

Part I of this thesis deals with the various imaging techniques, which are now available to visualize both clinical and subclinical forms of atherosclerosis. In chapter 1, we performed a literature study looking at the best methods to visualize atherosclerotic plaques in an early stadium both invasively and non-invasively. Here we compare non-invasive methods such as carotid intima-media thickness (IMT), Flow Mediated Dilation (FMD), multi-detector CT / electron beam CT and cardiac magnetic resonance imaging (MRI) with invasive methods such as quantitative coronary angiography (QCA) and intravascular ultrasound (IVUS). With regard to the non-invasive modalities, we show that IMT is the preferred method given the broadly validated technique, the simple applicability, the high degree of reproducibility and the ability to visualize both regression and progression of atherosclerosis. Among the invasive modalities, coronary angiography has been the gold standard for decades despite the fact that visualization thereof is limited to the arterial lumen and early stages of atherosclerosis can be missed easily. In our view, IVUS has the most potential to changes in atherosclerotic coronary arteries to visualize but the predictive value for future cardiovascular events for this modality should still be demonstrated. Yet, emerging techniques, including
320 multislice CT as well as MRI combined with functional measurements (either PET and/or molecular imaging techniques using selective ligands) are likely to enter the competition for optimal imaging technique. Currently, these techniques need further validation.

In chapter 2 we pay closer attention to the role of IMT as a surrogate marker for atherosclerosis. To that end, the main results of several key studies are discussed. First, results of observational studies show that the presence of cardiovascular disease (CVD) is positively associated with IMT. Another observational study of 4476 patients showed that this positive association between IMT and CVD also applies to asymptomatic patients. Furthermore, results from key statin intervention trials performed in various patient populations show that IMT enables evaluation of the effects of these drugs on the vessel wall. Some comments on the use of IMT are also made. First, the comparative value of IMT between different studies is limited due to lack of standardization of imaging protocols. More specifically, IMT has been shown to be especially valuable in patients with high IMT progression rate, in whom the change in LDL-C is linearly related to change in IMT: higher LDL decrease, less IMT progression. However, in patients already receiving optimal LDL lowering therapy and hence low IMT progression, the limited resolution of IMT may preclude to observe differential IMT progression in these patients (Enhance study).

In Part II of this thesis we shift our attention to LDL cholesterol, and its role in the process of atherosclerosis. In chapter 3, we start off with a discussion of the genetic backgrounds of a condition named Familial Hypobetalipoproteinemia (FHBL). This is a rare disorder, which is characterized by extremely low levels (less than the 5th percentile for age and gender) of apolipoprotein B (apoB) and plasma LDL-C. The condition results from mutations in the gene that encodes for apoB leading to truncated forms of apoB. New mutations are described for people who meet the above mentioned lipid criteria. The hypothesis is postulated that the extremely low LDL-C values would lead to less atherosclerosis and thus confer protection against the development of CVD. In chapter 4 the latter hypothesis was tested by measurement of the carotid IMT in 41 patients with FHBL and comparing these with measurements performed in 41 healthy control subjects. The observed slight difference in IMT in favor of the FHBL group lost significance after adjusting for traditional risk factors such as age, sex, smoking and body mass index. The small groups studied and the fact that the comparative arm consisted of healthy controls may partly explain this outcome in our opinion. It has recently become clear that the IMT technique is less suitable to detect less IMT progression compared to healthy controls. In fact, this most likely reflects the limited resolution of the IMT technique as discussed above. So, to demonstrate a decreased risk of CVD by means of a thinner IMT in FHBL subjects, as compared to control subjects, would require inclusion of a large number of subjects (> 750 patients). However, additional analysis using a cumulative risk score based on age, systolic blood pressure and smoking, did show significantly lower arterial wall stiffness in the FHBL group as compared to the healthy controls. Previous studies have shown that arterial wall stiffness is an
important predictor for future cardiovascular events. Our findings confirm that life-long ex- position of FHBL subjects to extremely low levels of LDL-C reduces the risk of developing atherosclerosis. In chapter 5 we show an overview of the accumulated clinical experience with a new cholesterol lowering drug called Inegy™. Inegy™ is a combination preparation of ezetimibe and simvastatin. Simvastatin belongs to the class of statins, which inhibit the production of cholesterol in the liver by inhibiting a key enzyme, the hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase. Ezetimibe belongs to a whole new class of cholesterol absorption inhibitors and inhibits the absorption of cholesterol in the intestine without affecting the uptake of triglycerides and fat-soluble vitamins. With Inegy™ it is possible to achieve LDL-C reductions up to 61% from baseline, which exceeds reductions achieved with statin monotherapy. Moreover, the data shows that ezetimibe is well tolerated by patients with little side effects reported by patients.

As briefly discussed above, the recent results of several clinical studies with Inegy™ have evoked much debate about the ‘simple’ concept of LDL reduction. In 2008 the annual congress of the American College of Cardiology called for a broad change in prescription behavior to the detriment of Ezetimibe-containing combination preparations. This was based on the ‘negative’ findings of Ezetimibe combination therapy on IMT in the ENHANCE study and also on the lack of additional benefit in the SEAS trial, which showed a disappointing reduction of cardiovascular risk in patients with aortic stenosis. Does this mean that Section 5 should be rewritten? In the personal view of this author, this is not the case. First of all, it is not surprising that in the ENHANCE trial, no further reduction in IMT could be demonstrated in Familial Hypercholesterolemia (FH) patients treated with simvastatin-combination therapy as compared to FH patients treated with simvastatin monotherapy. The study shows that, in contrast to previous studies such as the ASAP trial, FH patients already exhibited ‘thin’ IMT at baseline of the study. Secondly, the progression of IMT under the ‘standard’ treatment, simvastatin monotherapy, had already been halted. Consequently, it is theoretically impossible to inhibit progression better than stagnation. The main reason for this study outcome seems to be due to the inclusion of particularly well-treated FH patients with low disease load. In the SEAS study, patients with aortic valve stenosis were treated with simvastatin-ezetimibe versus placebo. The primary endpoint, a composite of major cardiovascular events, showed no significant difference. However, it is unknown whether lowering of LDL-C can actually influence this process. The relatively low reduction in ischemic cardiovascular events in treated patients is difficult to interpret since the study was not powered for this particular endpoint. In conclusion, LDL-C in patients should be reduced optimally, primarily by properly dosing statin therapy. If this strategy fails for whatever reason, adding Ezetimibe to the medication should be considered. In 2012, results of the IMPROVE IT trial will enable us to establish whether the combination of simvastatin-ezetimibe vs. simvastatin alone is actually better in reducing cardiovascular events as compared to treatment with statin monotherapy.
In Part III of the thesis we focus on another patient group that increasingly has to deal with atherosclerosis, the patients who are infected with the human immunodeficiency virus type 1 (HIV-1). The risks for CVD are discussed as well as the harmful but also potentially beneficial effects of antiretroviral therapy. Therapeutic options to combat CVD in this particular group of patients are also discussed.

In chapter 6 we start with an overview of the literature in which we indicate how infection with HIV-1 relates to CVD. Dyslipidemia in untreated HIV-1 infection is characterized by reduced high-density lipoprotein cholesterol (HDL-C) content. The inverse relationship between the level of HDL-C and the risk of CVD in the general population has been well validated in large epidemiological and clinical studies. HIV-1 infection is currently effectively suppressed by combining 3 or more antiretroviral drugs, often from different drug classes. This is called combination antiretroviral therapy or CART. We show that CART and, in particular HIV protease inhibitors (PI) containing CART, is associated with an increased risk of CVD. This is due to the metabolic complications of this therapy, such as dyslipidemia, insulin resistance and changes in body fat distribution (lipatrophy). Non-nucleoside reverse transcriptase inhibitors (NNRTIs) show exactly the opposite with increases in HDL-C and do not contribute to this risk of CVD. HIV-1 infection itself also seems to contribute to the increased risk of CVD, as shown for instance in autopsy studies of the pre-CART era in which extensive atherosclerotic lesions were found in the coronary arteries of young deceased patients. These abnormalities could not be explained by traditional risk factors for CVD. Furthermore, HIV-1 appears to intervene directly on one of the main functions of HDL-C, the reverse cholesterol transport. Reverse cholesterol transport is the process by which excess cholesterol from peripheral tissues is transported to HDL particles, which are subsequently delivered to the liver where they are taken up and excreted via bile acid and through the bile. The first step in this journey is the ABCA1-mediated cholesterol efflux from tissue macrophages to the HDL particles. In vitro studies show that HIV-1 has been able to interfere with this ABCA1-mediated efflux by down-regulation of ABCA1, which will lead to the accumulation of cholesterol in the macrophages and could promote accelerated development of atherosclerosis. In addition to these direct effects, it is shown that HIV-1 promotes the atherosclerosis process by means of chronic immune activation. Various coagulating factors are influenced leading to a hypercoagulabel status and it was shown that immune modulating therapy of HIV might limit the atherosclerotic plaques in animal studies. The accelerated atherosclerosis in HIV-1 patients is therefore based on both direct and indirect influences of the virus on the vessel wall, lipid profiles and clotting factors.

Chapter 7 outlines the various ways in which anti-retroviral drugs can affect plasma lipids. Using a unique population of HIV-1 negative neonates who were born from HIV-1 positive mothers and received prevention of HIV-1 transmission through breast milk for at least 3 months by administration of the NNRTI nevirapine (NVP) or the nucleoside reverse transcriptase inhibitor lamivudine (3TC). Previous studies using NVP in HIV-1 positive adults have already shown the
strong HDL-enhancing properties of this drug. In this study it became clear that plasma levels of HDL and apoA-I concentrations were increased to a greater extent in the NVP group as compared with the 3TC group. Results from this study show that the observed effects on HDL-C are not merely a return of HDL-C levels to “pre-infection values” but that intrinsic properties of NVP may be concerned. Indeed, the current study was conducted in HIV-1 negative neonates. The question remains whether the observed increase in plasma HDL-C levels is potentially beneficial in terms of the risk of CVD.

Chapter 8. To answer the preceding question we performed carotid IMT measurements in 62 HIV-1 patients who were treated with PI and compared this with the results of 68 HIV-1 patients who were treated with NVP or efavirenz, another NNRTI. Indeed, significantly higher HDL-C levels were observed in the group of patients treated with NNRTIs. The carotid IMT measurements were also lower (and thus more favorable) in the group treated with NNRTIs, which is expected to translate into a lower risk of CVD. Unfortunately, HDL-C levels were found not to be independent predictors of carotid IMT in the multivariate analysis. Apparently, factors other than HDL-C may also play a role.

In chapter 9, we tried to gain more insight into the mechanism behind the previously mentioned HDL-C increase with the use of the non-nucleoside reverse transcriptase inhibitor NVP. Within a 24 weeks study period 3 apoA-I kinetic tests were performed in 14 HIV-1 infected patients who were treated for at least 6 months with AZT/3TC/abacavir. Patients had to have an undetectable viral load (HIV-1 RNA <50 copies / ml) in the 6 months preceding inclusion. Key HDL-C modulating enzymes were also measured. We show that the addition of NVP to the treatment with AZT/3TC/abacavir leads to significant increases in plasma levels of HDL-C and apoA-I. This appears to be the effect of an increased production of apoA-I while catabolism of this protein remains unaffected. The significant increase in LCAT activity is low in absolute terms (9%) and therefore we believe it not to be an adequate explanation for the increase found in HDL-C/apoA-I levels. Recent results of the DAD study, a large multicenter prospective cohort study with clinical endpoints, show that the increased risk of CVD with HIV-1 patients is mainly related to the use of PI-containing CART and not to that of NNRTI-containing CART. In the SMART study therapy interruption of CART in HIV-1 patients was accompanied by a decrease in HDL-C levels, which was associated with a 2 times increased risk of developing CVD. The greatest decreases in HDL-C were observed in the patients treated with NNRTIs at the time of the interruption. These results suggest that higher HDL-C concentrations protect against the occurrence of CVD. The current study results may contribute to the development of future selective HDL-potentiators, not only for HIV-1 patients but also for patients from the general population. In the absence of potent and safe HDL-C raising drugs, the present therapy of PI-induced dyslipidemia in HIV-1 patients still focuses on the reduction of LDL-C. However, most statins are contraindicated because PI, including lopinavir/ritonavir, interferes with the metabolism
of statins by inhibition of the cytochrome P450. In chapter 10 we report the results from a pilot study comparing the efficacy and safety of rosuvastatin in HIV-1 patients with dyslipidemia caused by the PI lopinavir/ritonavir. Since rosuvastatin undergoes minimal metabolism via the cytochrome P450 enzyme it was thought that this statin would present a safe alternative for HIV-1 patients. As expected, the use of rosuvastatin was associated with strong reductions in plasma LDL-C levels. Contrary to expectations, increments in rosuvastatin plasma levels were observed for all dosages of rosuvastin. Based on this study we can conclude that cholesterol lowering with rosuvastatin is certainly possible in HIV-1 patients treated with PI-based CART. However, given the interaction between these drug classes, it seems wise to start with relatively low dosages, perform frequent checks of biochemical lab, and carefully inform patients about possible complications such as skeletal muscle toxicity (rhabdomyolysis). The current results are in line with the outcomes of previous studies investigating the interaction between PI and statins (other than rosuvastatin). Taken together, these results pose a powerful warning against liberal use of high dose statin therapy in this select group of patients.

**Future Prospects**

Techniques and methods for detection and modification of cardiovascular risk in patients with dyslipidemias, whether drug-induced or not, are continuously evolving on the basis of new scientific insights. The studies presented in this thesis argue for a high-throughput screening for cardiovascular disease using non-invasive imaging techniques. In particular, the one-stop-shop approach using MRI, in which structure, plaque composition and inflammation, as well as function (shear stress) can be visualized, is expected to present a revolutionary change in the risk assessment of individual patients.

With regard to LDL-C reduction, given the fact that most high-risk patients are already receiving aggressive LDL-lowering treatment it will become increasingly difficult to demonstrate further gains of novel interventions using the current surrogate markers. However, this notion should by no means be used as evidence of lack of effect in the assessment of investigational drugs, as this may lead to disqualification of potential favorable drugs. Clearly, further improvement of surrogate markers for CVD is desperately needed to assess the efficacy of novel therapeutics. Unlike the situation with respect to LDL lowering, HDL-enhancing strategies are still in their infancy. Moreover, HDL oriented research has received a heavy setback with the recent negative publicity concerning the CETP-inhibitor torcetrapib in patients at high cardiovascular risk. The increased morbidity and mortality in the torcetrapib arm appeared to be due to off-target toxicity of this compound. New modalities for selectively increasing HDL are essential to achieve auxiliary cardiovascular risk reduction in at risk patients. Our finding that NNRTIs may promote HDL production may contribute to further insights in the development of apoA-I production.
enhancers. Finally, we foresee that the contributory role of chronic inflammatory diseases in increasing the risk of CVD will become widely accepted within the next couple of years. With an ever-increasing incidence of CVD in the back of one's mind this added insight should guide doctors towards a policy change. This policy change would comprise improved screening for the presence of cardiovascular risk factors in patients suffering from inflammatory diseases such as HIV, rheumatoid arthritis, Crohn's disease and systemic lupus erythematosus. Presence of cardiovascular risk factors should prompt initiation of targeted risk-lowering therapy whenever possible.
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Samenvatting
Samenvatting

Atherosclerose is een chronisch ziekteproces van de arteriën welke zich afspelt op het niveau van de vaatwand. Cholesterol lekkage in de vaatwand resulteert in lokaal disfunctioneren van de vaatwand (endotheel disfunctie), wat gevolgd wordt door mobilisatie van monocyten naar de plek van endotheel disfunctie onder invloed van een toegenomen expressie van adhesie moleculen (met name VCAM-1) op endotheelcellen. Eenmaal in de subendotheliaal ruimte aangekomen, neemt de monocyt de eigenschappen van een macrofaag aan. Transformatie van deze macrofagen tot schuimcellen geschiedt door opname van subendotheliaal gelegen geoxideerde lipiden partikels. In de subendotheliaal ruimte is nu sprake van een ‘vaatwand’ ontsteking met als belangrijkste inflammatoire spelers de geactiveerde macrofagen en T-lymfocyten, die een continue bron van chemo-attractanten en pro-inflammatoire cytokines vormen. De hierop volgende geleidelijke toename van inflammatoire cellen, debris en cholesterol leidt tot een progressieve vernauwing van het bloedvat, de atherosclerotische plaque. Door proliferatie van gladde spiercellen over de plaque heen kan de laesie zich meestal stabiliseren. Echter, het fibroze kapsel om de plaque heen kan ook ruptureren onder invloed van matrix metallo proteinases welke door de geactiveerde macrofagen geproduceerd worden. Ruptuur van de plaque gaat gepaard met het vrijkomen van tissue factor en leidt tot acute trombose- ring van het bloed ter plaatse met als gevolg een occlusie van het bloedvat. Langdurige occlusie van het bloedvat kan leiden tot een myocardinfarct (indien het een coronair arterie betreft) of een herseninfarct (als het een cerebrale arterie betreft). Verlaging van het LDL-C door middel van medicamenteuze behandeling met statines kan verdere aangroei van een plaque remmen en bij zeer agressieve verlaging van het LDL-C soms zelfs leiden tot regressie van de atherosclerotische plaques. Ook als primaire preventie voor hart- en vaatziekten zijn statines inmiddels zeer waardevol gebleken.

Deel I van dit proefschrift handelt over de diverse beeldvormende technieken die thans beschikbaar zijn om zowel klinische vormen als subklinische vormen van atherosclerose te visualiseren.

In hoofdstuk 1 hebben wij middels een literatuur studie gekeken naar de beste methoden, zowel invasief als non-invasief, om atherosclerotische plaques in een vroeg stadium te kunnen visualiseren. Hierbij zijn de non-invasieve methoden zoals carotis intima-media dikte (IMT), flow mediated dilation (FMD), multidetector CT/electron beam CT en de cardiale magnetische resonantie imaging (MRI) vergeleken met invasieve methoden zoals quantitatieve coronair angiografie (QCA) en de intravasculaire ultrasone (IVUS). Wij laten zien dat bij de non-invasieve modaliteiten IMT vooralsnog de voorkeur geniet boven de andere technieken vanwege de solide wetenschappelijke onderbouwing, de makkelijke toepasbaarheid, de grote mate van reproduceerbaarheid en het vermogen om zowel progressie als regressie van atherosclerose te visualiseren. Bij de invasieve modaliteiten wordt coronair angiografie al decennia tot de gou-
den standaard gerekend, ondanks het feit dat de visualisatie beperkt is tot het arteriële lumen en vroege stadia van atherosclerose dus gemakkelijk gemist kunnen worden. In onze optiek heeft IVUS de meeste potentie om atherosclerotische veranderingen in coronaire arteriën te visualiseren maar de predictieve waarde voor toekomstige cardiovasculaire events moet voor deze modaliteit nog aangetoond worden. Bovendien zullen opkomende technieken, zoals 320 multislice CT en MRI gecombineerd met functionele metingen (hetzij PET en/of moleculaire beeldvormende technieken met gebruikmaking van selectieve ligands) zullen waarschijnlijk in de nabije toekomst beschikbaar komen voor klinische toepassing en daarmee serieuze candidaten worden in de competitie voor de beste beeldvormende modaliteit. Momenteel wordt gewerkt aan validatie van de laatstgenoemde 2 technieken.

In hoofdstuk 2 wordt nader ingegaan op de rol van IMT als surrogaat marker voor atherosclerose. Hiertoe worden de belangrijkste resultaten van diverse sleutel studies besproken. Allereerst wordt aan de hand van observationele studies geïllustreerd dat de aanwezigheid van hart- en vaatziekten (HVZ) positief geassocieerd is met de dikte van IMT. Een andere observationele studie met 4476 patiënten liet zien dat deze positieve associatie tussen IMT en HVZ ook van toepassing is op asymptomatische patiënten. Voorts wordt aan de hand van voorname statine interventie trials bij diverse patiënten populaties aangetoond dat IMT heel goed in staat is om de effecten van deze medicijnen op de vaatwand te beoordelen. Kanttekeningen bij de toepassing van IMT worden echter ook gemaakt. Ten eerste, de vergelijkbaarheid van IMT uitkomsten tussen studies onderling wordt beperkt door een gebrek aan standaardisatie van beeldvormingsprotocollen. Bovendien blijkt IMT de grootste waarde te hebben bij patiënten die een snelle progressie van IMT doormaken, dat wil zeggen patiënten waarbij de verandering in LDL-C lineair gerelateerd is aan verandering in IMT. Hierbij gaat een sterke LDL-C reductie dus gepaard met minder IMT-progressie. Echter, bij tevoren reeds optimaal behandelde patiënten, en dientengevolge een laag LDL-C met een trage IMT progressie, zal de beperkte resolutie van IMT het onmogelijk maken om verdere verschillen in IMT progressie aan te tonen bij toevoeging van additionele cholesterol verlagers (Enhance studie).

In hoofdstuk 3 werden hiertoe eerst de genetische achtergronden belicht bij Familiaire Hypobetalipoproteinemie (FHBL). Dit is een zeldzame stoornis die gekenmerkt wordt door extreem verlaagde waarden (< 5e percentiel voor leeftijd en geslacht) van het apolipoproteine B (apoB) en het plasma LDL-cholesterol ten gevolge van mutaties in het gen dat codeert voor apoB. Nieuwe genmutaties worden beschreven bij mensen die voldoen aan dit lipidenprofiel. De hypothese wordt geopperd dat de extreem lage LDL-C waarden tot minder atherosclerose aanleiding zullen geven en aldus beschermend zouden zijn voor het ontwikkelen van HVZ.

In hoofdstuk 4 werd deze laatste hypothese getoetst door de carotis IMT te meten bij 41 patiënten met FHBL en dit te vergelijken met 41 gezonde controle patiënten. Het geringe ver-
In hoofdstuk 5 tonen wij een overzicht van de opgebouwde klinische ervaring met een nieuwe cholesterol verlager genaamd Inegy™. Inegy™ is een combinatie preparaat van ezetimibe en simvastatine. Simvastatine behoort tot de klasse van statines, deze remmen de aanmaak van cholesterol in de lever door remming van een essentiële enzym, het hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase. Ezetimibe behoort tot de geheel nieuwe klasse van cholesterol absorptie remmers en remt de opname van cholesterol in de darm zonder de opname van triglyceriden en vet oplosbare vitaminen te beïnvloeden. Met Inegy™ is het mogelijk om LDL-C reducties tot 61% te bereiken, meer dan met statine monotherapie mogelijk is. Verder blijkt uit de getoonde data dat het geneesmiddel goed verdragen wordt door patiënten en dat het weinig bijwerkingen vertoond.

Zoals hierboven reeds kort genoemd hebben de recente uitkomsten van diverse klinische studies met Inegy™ echter veel stof doen opwaaien omtrent het ‘eenvoudige’ concept van LDL verlaging. Op de jaarlijkse conferentie van de American College of Cardiology in 2008 werd zelfs gevraagd om een brede wijziging van prescriptie gedrag ten nadele van ezetimibe-bevattende combinatie preparaten. Dit laatste berust enerzijds op de ‘negatieve’ bevindingen van ezetimibe combinatie therapie op IMT in de ENHANCE studie en anderzijds op het gebrek aan toegevoegde waarde in de SEAS trial met een tegenvallende reductie van cardiovasculaire events bij patiënten met een aorta stenose. Betekent dit dat hoofdstuk 5 herschreven zou moeten worden? In de persoonlijke optiek van deze auteur geenszins. Allereerst is het niet verwonderlijk dat de ENHANCE trial geen verdere reductie van IMT kon aantonen in (Familiaire Hypercholesterolemie) FH patiënten behandeld met simvastatine-ezetimibe combinatie therapie ten opzichte van simvastatine monotherapie aangezien de FH patiënten bij inclusie reeds ‘dunne’ IMTs hadden, dit in tegenstelling tot eerdere studies in FH patiënten zoals...
de ASAP studie. Ten tweede was de progressie van IMT onder de ‘standaard’ behandeling, dus simvastatine monotherapie, reeds totaal te niet gedaan. Dientengevolge is het theoretisch al onmogelijk progressie beter te remmen dan stilstand. De belangrijkste reden van deze studie uitkomst lijkt dan ook te wijzen aan de inclusie van met name goed behandelde FH patiënten met een lage ziekte-load.

In de SEAS studie werden patiënten met een aortaklep stenose behandeld met de combinatie van simvastatine-ezetimibe versus placebo. Het primaire eindpunt, een composiet van “major cardiovascular events”, toonde geen significant verschil. Echter, het is onbekend of LDL-C verlaging dit proces daadwerkelijk kan beïnvloeden. De relatief lage reductie in ischemische cardiovasculaire events is moeilijk te duiden aangezien de studie niet gepowered was voor dit specifieke eindpunt. Concluderend dient het LDL-C bij patiënten optimaal gereduceerd te worden, primair met adequaat gedoseerde statine therapie. Als dit om wat voor reden dan ook onvoldoende lukt kan overwogen worden ezetimibe aan de medicatie toe te voegen. In 2012 kan op basis van de uitkomsten van de IMPROVE IT trial definitief vastgesteld worden of deze combinatie ook daadwerkelijk het aantal hart- en vaatziekten verder reduceert in vergelijking tot statine monotherapie.

In deel III van het proefschrift richten wij ons op een andere patientengroep die steeds meer met atherosclerose te maken krijgt, de patiënten die geïnfecteerd zijn met het humaan immunodeficientie virus type 1 (HIV-1). De risico’s op HVZ worden besproken alsook de schadelijke maar ook potentieel gunstige effecten van antiretrovirale therapie. Therapeutische opties ter bestrijding van HVZ bij deze patientengroep passeren de revue.

In hoofdstuk 6 starten wij met een overzicht van de literatuur waarin wij aangeven hoe infectie met HIV-1 gerelateerd is aan HVZ. Dyslipidemie bij een onbehandelde HIV-1 infectie kenmerkt zich onder andere door een verlaagd high-density lipoprotein cholesterol (HDL-C) gehalte. De inverse relatie tussen de hoogte van het HDL-C en het risico op HVZ in de algemene bevolking is inmiddels door vele grote epidemiologische en klinische studies aangetoond. HIV-1 wordt tegenwoordig effectief bestreden door 3 of meer antiretrovirale medicijnen, veelal uit verschillende klassen, te combineren. Dit wordt combinatie antiretrovirale therapie of kortweg cART genoemd.

We laten zien dat cART, en in het bijzonder HIV-protease remmers (PI) bevattende cART, geassocieerd is met een verhoogd risico op HVZ. Dit komt door de metabole complicaties van deze therapie, zoals dyslipidemie, insuline resistentie en veranderingen in de lichaamsvet verdeling (lipoatrofie). Non-nucleoside reverse transcriptase remmers (NNRTI) blijken juist het HDL-C te verhogen en niet bij te dragen aan dit risico op HVZ. HIV-1 infectie zelf lijkt echter ook bij te dragen aan het verhoogd risico op HVZ zoals onder andere blijkt uit obductie studies van voor het cART tijdperk waarbij uitgebreide atherosclerotische laesies gevonden werden in de coronair arteriën van jong overleden patiënten. Deze afwijkingen konden niet verklaard worden door
klassieke risico factoren voor HVZ. Daarnaast blijkt HIV-1 rechtstreeks in te grijpen op een van de belangrijkste functies van HDL-C, het reverse cholesterol transport. Reverse cholesterol transport is het proces waarbij het overmaat aan cholesterol vanuit perifere weefsels getransporteerd wordt naar HDL partikels die het vervolgens naar de lever transporteren alwaar het via de gal wordt uitgescheiden. De eerste stap in dit transport is de ABCA1 gemedieerde efflux van cholesterol vanuit weefselmacrophagen naar de HDL-partikels. HIV-1 is in vitro in staat gebleken om deze ABCA1 gemedieerde efflux te remmen door down-regulatie van ABCA1 wat de accumulatie van cholesterol in de macrofagen en versneld optreden van atherosclerose zou kunnen bevorderen. Naast deze directe effecten blijkt HIV-1 ook door middel van chronische immuunactivatie het proces van atherosclerose te bevorderen. Zo worden diverse stollingsfactoren beïnvloed leidend tot een hypercoagulabele status en blijkt immunomodulerende therapie van HIV het ontstaan van atherosclerotische plaqes te remmen in dierexperimenten. Het versneld optreden van atherosclerose bij HIV-1 patiënten blijkt dus te berusten op zowel directe als indirecte invloeden van het virus op de vaatwand, lipidenprofielen en stollingsfactoren.

In hoofdstuk 7 wordt nader ingegaan op de verschillende wijzen waarop antiretrovirale middelen plasma lipiden kunnen beïnvloeden. Gebruikmakend van een unieke populatie van HIV-1 negatieve neonaten die geboren werden uit HIV-1 positieve moeders en ter preventie van HIV-1 transmissie via de moedermelk gedurende tenminste 3 maanden behandeld werden met de NNRTI nevirapine (NVP) of de nucleoside reverse transcriptase remmer lamivudine (3TC). NVP liet in eerdere studies bij HIV-1 positieve volwassenen reeds zien krachtige HDL-verhogende eigenschappen te bezitten. In deze studie werd duidelijk dat de plasma HDL spiegels en ook apoA-I concentraties sterker verhoogd waren in de NVP groep dan in de 3TC groep. Hiermee maken wij waarschijnlijk dat het bij eerdere studies gevonden effect van NVP op HDL-C niet puur en alleen te maken heeft met een stijging van de spiegels naar “pre-infectie waarden” maar dat het mede een intrinsieke eigenschap van NVP betreft. Immers, de huidige studie werd uitgevoerd bij HIV-1 negatieve neonaten. De vraag blijft echter of de gevonden HDL-C stijging potentieel gunstig is wat betreft het risico op HVZ.

Om deze voorgaande vraag te beantwoorden hebben wij in hoofdstuk 8 carotis-IMT metingen verricht bij 62 HIV-1 patiënten die met PI behandeld werden en dit vergeleken met de uitkomsten bij 68 HIV-1 patiënten die met NVP of efavirenz, een andere NNRTI, behandeld werden. Hierbij vonden wij inderdaad significant hogere HDL-C spiegels bij de groep patiënten die behandeld werd met NNRTI. De carotis IMT metingen bleken ook lager (en dus gunstiger) te zijn in de groep die behandeld werd met NNRTI wat zich naar verwachting zou kunnen vertalen in een lager risico op HVZ. Helaas bleken HDL-C spiegels in de multivariaat analyse geen onafhankelijke voorspellers voor carotis IMT te zijn. Kennelijk spelen andere factoren dan het HDL-C mede een rol.
In **hoofdstuk 9** hebben wij getracht meer inzicht te krijgen in het mechanisme achter de eerder genoemde HDL-C stijging bij gebruik van de non-nucleoside reverse transcriptase remmer NVP. Hiervoor werden in een periode van 24 weken 3x apoA-I kinetiek proeven verricht bij 14 HIV-1 geïnfecteerde patiënten die tenminste 6 maanden behandeld werden AZT/3TC/abacavir en gedurende die periode een niet detecteerbare viral load (HIV-1 RNA <50 copies/ml) hadden. Belangrijke HDL-C modulerende enzymen werden eveneens gemeten. Wij laten zien dat toevoeging van NVP aan de behandeling met AZT/3TC/abacavir leidt tot significante verhogingen van de plasma spiegels van HDL-C en apoA-I. Dit hangt samen met een verhoogde productie van apoA-I zonder dat het catabolisme van dit eiwit beïnvloed wordt. De significante verhoging in LCAT activiteit is in absolute zin laag (9%) en onzes inziens daarom geen afdoende verklaring voor de gevonden stijging in HDL-C/apoA-I spiegels. Recente uitkomsten van de DAD studie, een grote multicenter prospectieve cohort studie met klinische eindpunten, laten zien dat het verhoogd risico op HVZ bij HIV-1 patiënten vooral gerelateerd is aan het gebruik van PI-bevattende cART en niet aan dat van NNRTI-bevattende cART. In de SMART studie werd aangetoond dat therapie interruptie van cART bij HIV-1 patiënten gepaard ging met een daling van het HDL-C en dat het geassocieerd was met een 2x verhoogd risico op het ontwikkelen van HVZ. De HDL-C dalingen bleken bovendien het grootst te zijn bij de patiënten die met NNRTI behandelld werden ten tijde van de interruptie. Deze uitkomsten suggereren dat hogere HDL-C concentraties beschermen tegen het optreden van HVZ. Mogelijk dat onze studie uitkomsten kunnen bijdragen aan de ontwikkeling van toekomstige selectieve HDL-C verhogers, niet alleen voor HIV-1 patiënten maar ook voor patiënten uit de algemene populatie.

Bij gebrek aan krachtige en veilige HDL-C verhogers richt de huidige therapie van PI-geinduceerde dyslipidemie bij HIV-1 patiënten zich nog steeds op verlaging van het LDL-C. De meeste statines zijn echter gecontraindiceerd omdat PI, inclusief lopinavir/ritonavir, het metabolisme van statines remmen door remming van het cytochrom P450. In **hoofdstuk 10** doen wij verslag van een pilot studie waarbij de effectiviteit en veiligheid van rosuvastatine wordt onderzocht bij HIV-1 patiënten met een dyslipidemie veroorzaakt door de PI lopinavir/ritonavir. Aangezien rosuvastatine slechts minimale metabolisering ondergaat via het cytochrom P450 enzym was de gedachte dat dit een veilige combinatie zou zijn voor HIV-1 patiënten. LDL-C concentraties bleken krachtig gereduceerd te worden bij gebruik van rosuvastatine. Een belangrijk punt van aandacht verdient echter wel de rosuvastatine spiegel stijging die bij alle doseringen van rosuvastine werd waargenomen. Op basis van deze studie kan geconcludeerd worden dat rosuvastatine therapie bij cART therapie (lopinavir/ritonavir) zeker mogelijk is. Echter, gezien de interactie tussen de medicatie vormen lijkt het verstandig relatief laag te doseren, frequente biochemische controes te verrichten en goede voorlichting te geven over de mogelijkheid van spier complicaties (rhabdomyolyse). De huidige resultaten zijn in lijn met de uitkomsten van eerdere interactie studies tussen PI en de overige statines. Tezamen vormen zij een krachtige waarschuwing tegen liberaal gebruik van hoge dose statine therapie in deze groep patiënten.
Toekomstperspectieven

De thans beschikbare technieken en methoden ter detectie en modificatie van het cardiovasculair risico bij patienten met, al dan niet medicamenteus geïnduceerde, dyslipidemieën zullen zich steeds verder ontwikkelen op basis van voortschrijdende wetenschappelijke inzichten. De studies gepresenteerd in dit proefschrift pleiten voor een high throughput screening voor hart- en vaatziekten met behulp van non-invasieve beeldvormende technieken. Met name de one-stop-shop benadering met MRI, waarbij in 1 onderzoek zowel structuur, plaque composietie en inflammatie, alsmede functie (shear stress) kan worden gevisualiseerd, zal naar verwachting een revolutionaire verandering gaan vormen bij de individuele risicoschatting van patienten.

Wat LDL verlaging betreft zal het gezien de huidige graad van therapie steeds moeilijker worden om verdere winst voor patiënten aan te tonen in surrogaat marker studies. Echter, dit dient niet zo maar gebruikt te worden als bewijs van geen effect bij de beoordeling van nieuwe geneesmiddelen, omdat hier anders veelbelovende medicijnen mogelijk ten onrechte het etiket onwerkzaam opgeplakt zullen krijgen. Ook hier is verdere verbetering van surrogaat markers dus noodzakelijk.

In tegenstelling tot de situatie met betrekking tot LDL verlaging staat het onderzoek met betrekking tot HDL verhogende strategieën nog relatief in de kinderschoenen. Dit onderzoek heeft bovendien een zware klap gekregen door onder andere de recente verwikkelingen rond de CETP-remmer Torcetrapib, hetgeen echter met name op off-target toxicity van dit middel bleek te berusten.

Zo bleek de oversterfte bij gebruik van torcetrapib samen te hangen met inductie van hyperaldosteronisme en hypokaliemie. Nieuwe modaliteiten om selectief HDL te verhogen zijn essentieel voor een verdere reductie van het cardiovasculair risico. Onze bevinding dat NNRTIs HDL productieijken aan te zetten kunnen mogelijk een bijdrage leveren aan verdere inzichten bij de ontwikkeling van apoAI-productie enhancers.

De rol van chronische inflammatoire ziekten bij het verhogen van het cardiovasculair risico zal niet alleen algemeen geaccepteerd moeten worden, maar mede gezien de toenemende incidentie van HVZ tegelijkertijd ook moeten leiden tot een beleidswijziging bij artsen. Zo zullen patienten die leiden aan ziekten als HIV, reumatoïde artritis, de ziekte van Crohn en systemische lupus erythematosus niet alleen beter gescreeid moeten worden op de aanwezigheid van cardiovasculaire risicofactoren maar zal de aanwezigheid daarvan, waar mogelijk, aanleiding moeten zijn tot het starten van gerichte risico verlagende therapie.
Dankwoord
Dankwoord

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Atherosclerosis in the HIV and Non-HIV setting: detecting and modifying cardiovascular risk

1. De uitkomsten van studies die gebruik maken van surrogaat markers hoeven niet altijd juist te zijn (dit proefschrift).


3. Soms kan een laag LDL-cholesterol slecht voor de lever zijn! (dit proefschrift).


5. Nevirapine, een non-nucleoside reverse transcriptase remmer, is in meerdere opzichten levensreddend (dit proefschrift).

6. Niets maakt spaarders ongeruster dan een Minister van Financiën die meldt dat “spaarders zich niet ongerust hoeven te maken”.

7. An educated person is one who has learned that information almost always turns out to be at best incomplete and very often false, misleading, fictitious, mendacious - just dead wrong (Russell Baker).

8. Science is facts; just as houses are made of stones, so is science made of facts; but a pile of stones is not a house and a collection of facts is not necessarily science (Henri Poincare).

9. An expert is a man who has made all the mistakes which can be made in a very narrow field (Niels Bohr).

10. Men are born to succeed, not fail (Henry David Thoreau).