Efficacy, safety and novel targets in cardiovascular disease : advanced applications in APOE*3-Leiden.CETP mice

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The BCR-ABL1 tyrosine kinase inhibitors imatinib and ponatinib decrease plasma cholesterol through different effects on lipoprotein metabolism

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Abstract

Objectives: Chronic myeloid leukemia (CML) is treated with BCR-ABL1 tyrosine kinase inhibitors (TKIs), but modulations in plasma lipids occur. The objectives of this study were to evaluate the effect of three generations TKIs on plasma cholesterol and triglyceride (TG) metabolism and to investigate the underlying mechanism using APOE*3-Leiden.CETP mice, a model that mimics human lipoprotein metabolism.

Methods and results: Mice were fed a Western-type diet and were treated for 6 weeks with either imatinib, nilotinib or ponatinib at drug exposures relevant to CML patients. The effects on plasma and liver lipids, lipoprotein metabolism, and fecal lipid excretion were assessed. Imatinib decreased plasma non-high-density lipoprotein cholesterol (non-HDL-C) (-52%) and TG (-42%) mainly by reducing VLDL-TG and VLDL-apolipoprotein-B production, and reduced cholesterol ester (CE) content of the VLDL particles. This was accompanied by a reduction in the majority of the lipid classes (triacylglycerols, CEs, glycerophospholipids), including the pro-atherogenic sphingolipids, as determined by lipidomics analysis. Ponatinib decreased plasma non-HDL-C levels (-26%) by lowering intestinal cholesterol absorption. Moreover, ponatinib reduced the CE content in the liver and in the VLDL particles. Nilotinib did not affect lipoprotein metabolism.

Conclusions: Our data confirm the lipid-lowering effects of imatinib in CML-patients and provide an explanation by showing that imatinib and ponatinib affect lipoprotein metabolism through distinct mechanisms.

Introduction

BCR-ABL1 tyrosine kinase inhibitors (TKIs) are the standard of care for treatment of chronic myeloid leukemia (CML). The first line TKI imatinib is effective and safe, but not all patients do have a complete cytogenetic response or develop drug resistance. Consequently, novel and more potent TKIs have been developed, e.g. nilotinib and ponatinib, the latter as the only TKI with activity against the T315I mutation. Unfortunately, cardiovascular safety issues including ischemic heart disease and progressive arterial occlusive disease (PAOD) have been reported with nilotinib and ponatinib (1–4), which preferentially developed in those patients having a (very) high cardiovascular risk according to the SCORE chart (5,6).

Several studies describe plasma lipid modulations in CML-patients during TKI treatment. Imatinib consistently decreases plasma cholesterol, and even normalization in hypercholesterolemic CML-patients has been reported (7–10). Retrospective analysis of phase III studies revealed a lower incidence of cardiovascular events in patients treated with imatinib relative to patients treated without TKIs (11). In contrast, several studies with nilotinib reported increased plasma cholesterol levels (1,10,12,13), although these findings are not consistent (14). Data on ponatinib are scarce but one study reported no alterations in plasma lipids (10).

It is worth noting that in the setting of CML, both indirect effects of the underlying disease as well as direct effects of TKI-treatment may modulate cardiovascular risk factors, including plasma lipid levels. Therefore, we have previously investigated the (patho) physiology of the decreased cardiovascular risk by imatinib and the increased cardiovascular risk by nilotinib and ponatinib using a mouse model without CML, the APOE*3-Leiden.CETP mouse (15). At similar drug exposures as in CML-patients, we found that imatinib reduced plasma cholesterol and triglyceride (TG) levels, decreased atherosclerotic lesion size and improved lesion stability, all in line with the reported lipid reductions and improved cardiovascular outcome in CML-patients during imatinib treatment (7–11). Furthermore, ponatinib reduced plasma cholesterol levels and atherosclerosis progression, whereas nilotinib did not affect plasma lipid levels or atherosclerosis. Interestingly, nilotinib and ponatinib adversely affected genes involved in coagulation and increased plasma levels of FVII (ponatinib) and FVIIa (nilotinib) (15), important factors in the pathogenesis of atherothrombotic events. These findings suggest that not enhanced atherosclerosis progression, but changes in coagulation are related to the observed cardiotoxicity by nilotinib and ponatinib.

The lipid-modulating effects of imatinib are well described, but there are no reports that provide a mechanistical explanation for the observed effects. Also, little is known about the ability of ponatinib to affect lipoprotein metabolism, and the inconsistent effects of nilotinib on plasma lipids in CML-patients require further investigation. Therefore, the aim of this study was to investigate the mechanism underlying the ability of these TKIs

to affect lipid homeostasis. As in our previous study, we used the APOE*3-Leiden.CETP mouse model, since these mice, like humans, have a delayed clearance of apolipoprotein-B (apoB)-containing lipoproteins and express cholesteryl ester transfer protein (CETP), resulting in a lipoprotein profile similar as in patients with familial dysbetalipoproteinemia, and a human-like lipoprotein metabolism (16). This mouse model responds to all hypolipidemic drugs used in the clinic similarly as patients (17–19) and has been widely used to study the effect of drugs and other compounds on atherosclerosis (18–21), lipoprotein metabolism (22–26) and cardiovascular safety (27), including our previous study with BCR-ABL1 inhibitors (15).

Materials and methods

Animals

Eighty female APOE*3-Leiden.CETP transgenic mice on a C57BL/6 background (9 - 12 weeks of age) were obtained from the breeding facility of the Netherlands Organization of Applied Scientific Research (TNO), Leiden, the Netherlands. In this study, 4 groups of 16 - 17 mice were used, and per treatment group the mice were divided into two groups of each 8 - 9 mice as two different endpoint experiments were performed (**Table 1**). The number of mice was based on our experience from previous studies and was calculated using a probability of 0.05. We expected to have a variance of 18% in the endpoint experiments (VLDL clearance or VLDL production), a minimal difference of 50% and a two-sided test with 95% confidence interval, which resulted in 8 animals per experiment. Since, it is known that approximately 20% of the APOE*3-Leiden.CETP mice do not respond properly to the Western-type diet with respect to increasing their plasma cholesterol (TC) and TG (i.e. low-responders), the study initially started with 80 mice. Mice were fed a semi-synthetic diet, containing 15% (w/w) saturated fat from cacao butter and 0.15% (w/w) cholesterol (Western-type diet [WTD]; Altromin, Tiel, the Netherlands) for 3 weeks, and subsequently the low-responder mice were selected based on TC and TG levels and removed from the study prior to allocation into groups (**Table 1**). Since there were less low-responders as predicted, 67 mice were randomized according to body weight, age, plasma TC, and TG levels in 4 groups of 16 - 17 mice. The mice entered the study in a staggered way of 1 week apart with two batches of each 8 - 9 mice per group. During the study, mice were group-housed (4 - 5 mice per cage) under standard conditions with a 12-h light-dark cycle and had free access to food and water. Body weight, food intake and clinical signs of discomfort were monitored regularly during the study. The care and use of all mice in this study was carried out at the animal facility of TNO in accordance with national and EU ethical regulations. Animal experiments were approved by the Institutional Animal Care and Use Committee of TNO under registration number 3682.

Table 1 Study design

Time (weeks)	**All mice (n = 80)**	
-3 to 0	Run-in/acclimatization on a Western type diet	
0	Selection and exclusion of low-responders (n = 13)	
0	Matching in 4 groups based on plasma cholesterol, triglycerides, age and body weight	
Time (weeks)	**In life phase (4 groups of 16 - 17 mice)**	
0, 3, 6	Body weight, food intake and plasma parameters	
5	Fecal neutral sterols and bile acids	
5	Cholesterol absorption	
6	Lipidomics	
Time (weeks)	**End-experiment 1 (n = 8 per group)**	**End-experiment 2 (n = 8 - 9 per group)**
6	VLDL clearance	VLDL production
	Hepatic lipid content	

After a run-in period of 3 weeks on a Western-type diet mice were matched in 4 groups of each 16 17 mice and were treated for 6 weeks with 3 generations TKIs, imatinib (150 mg/kg BID), nilotinib (30 mg/kg QD for the first 3 weeks and 10 mg/kg QD during the last 3 weeks) or ponatinib (10 mg/kg QD first 3 weeks and 3 mg/kg QD last 3 weeks). After 6 weeks, mice were divided into 8 - 9 mice per group per experiment to assess VLDL clearance and VLDL production. Abbreviations: VLDL, very-low-density-lipoprotein

Experimental design

Upon randomization, mice received, based on the results of a previous pharmacokinetic (PK) study (15), an once-daily oral gavage with nilotinib (30 mg/kg), ponatinib (10 mg/kg), or a twice-daily gavage with imatinib (150 mg/kg) for 3 weeks to confirm our previous findings on plasma lipids (15), after which the doses were reduced for nilotinib (10 mg/kg) and ponatinib (3 mg/kg) to match better with relevant human doses (15). Doses and dose intervals were based on data obtained from a previously performed PK study (15). All The TKIs were suspended in 5% carboxymethyl cellulose (CMC) and all mice except the imatinib group received a second oral gavage with the vehicle (5% CMC). Body weight, food intake, plasma TC, TG, and high-density lipoprotein-cholesterol (HDL-C) were measured throughout and non-HDL-C was calculated by subtracting HDL-C from TC. Feces were collected in week 5 for the determination of bile acids and fecal neutral sterols. Lipidomics analysis was carried out in 4-hour fasted plasma of 8 mice per treatment group from week 6. After 6 weeks of treatment, two different endpoint experiments were performed: (I) very-low density-lipoprotein (VLDL)-like particle clearance was determined in 8 mice per treatment group, and (II) VLDL production and *de novo* apoB synthesis was

assessed in 8 - 9 mice per treatment group as described previously (26). Hepatic lipid content was analyzed in mice of experiment 1 (**Table 1**).

Plasma biochemical analysis

Plasma samples were collected in week 0, 3 and 6 after a 4-hour fast. Plasma TC and TG were determined using enzymatic kits (TC: Roche/Hitachi, catalogue# 11491458216, TG: Roche/Hitachi, catalogue# 11730711216) according to the manufacturer's protocols. HDL-C was measured after precipitation as described previously (21). Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) enzymatic activity was measured by reflectance photometry using a Reflotron® Plus analyzer (Hoffman-La Roche, Basel, Switzerland).

Excretion of fecal sterols and bile acids

Fecal excretion of bile acids and neutral sterols was determined in feces, collected per cage during a 48- to 72-hour time period at three consecutive time points at week 5, by gas chromatographic analysis as described by Post et al (28).

In vivo clearance of VLDL-like particles

Mice (8 per treatment group) were fasted for 4 hours and injected in the tail vein with VLDL-like particles (80 nm) containing 3H-labelled fatty acids (FA) (as glycerol tri[3H]-oleate, [3H]-TO) and 14C-labelled cholesteryl oleate (as [14C]-cholesteryl oleate, [14C]-CO). At t=2, 5, 10 and 15 minutes post-injection, blood was collected to determine the plasma decay of [3H]-TO and [14C]-CO. At 15 minutes, mice were euthanized by cervical dislocation and perfused with heparin 10 U/mL in ice-cold PBS for 5 minutes. Organs (i.e. small intestine, right kidney, heart, spleen, lung, brown adipose tissue (BAT), gonadal white adipose tissue (gWAT), subcutaneous WAT (sWAT), femoral muscle and liver) were harvested and saponified overnight in 500 µl Solvable (Perkin-Elmer, Wellesley, MA) to determine [3H]-TO and [14C]-CO uptake. Retention of radioactivity in the saponified tissues was measured as % of the injected dose, and the half-life of VLDL-[3H]-TO and VLDL-[14C]-CO was calculated from the slope after linear fitting of semi-logarithmic decay curves as described previously (22,25,26,29).

Hepatic VLDL-TG and VLDL-apoB production

Mice (8 - 9 per treatment group) were fasted for 4 hours prior to the start of the experiment. During the experiment, mice were sedated with acepromazine-midazolam-fentanyl intraperitoneally [6.25 mg/kg acepromazine (Ceva Santé Animale), 6.25 mg/kg midazolam (Actavis), and 0.3125 mg/kg fentanyl (Bipharma)]. At t=0 minutes, blood was taken via tail bleeding and mice were intravenously (IV) injected with 100 µl phosphate buffered saline (PBS) containing 20 µCi Trans[35S]-labelled methionine/cysteine (ICN Biomedicals, Irvine, CA) to measure *de novo* apoB synthesis. After 30 minutes, the mice received a Triton WR1339 IV injection (500 mg/kg body weight), which inhibits lipoprotein lipase (LPL)

mediated lipolysis, thereby blocking VLDL clearance. Blood samples were drawn at 0, 15, 30, 60 and 90 minutes after Triton WR1339 injection and used for determination of the plasma TG concentration. After 90 minutes, the animals were sacrificed by cervical dislocation and blood was collected by heart puncture for subsequent isolation of VLDL by density-gradient ultracentrifugation. [35S]-apoB was measured in the VLDL fraction after apoB-specific precipitation, and VLDL-apoB production rate was calculated as disintegration per minute (dpm)/h, as previously reported (22,25,26,29). The free cholesterol (FC), cholesterol ester (CE), TG and phospholipid (PPL) content of the VLDL particles was determined using the kits "Cholesterol CHOD-PAP" (Roche, Mannheim, Germany), "Free cholesterol E" (Instruchemie, Delfzijl, the Netherlands), "Triglycerides GPO-PAP" (Roche, Mannheim, Germany), and "Phospholipids" (Instruchemie, Delfzijl, the Netherlands), respectively.

Hepatic lipid analysis

Frozen liver tissue samples of lobus sinister lateralis hepatis were homogenized at 4°C in phosphate-buffered saline, and the protein content was measured using a Lowry protein assay. Lipids were extracted, separated by high-performance thin-layer chromatography on silica gel plates, stained as described previously (30), and analyzed with ChemiDoc Touch Imaging System (Bio-Rad). TG, CE and FC content were quantified using Image-lab version 5.2.1 software (Bio-Rad) and expressed per mg liver protein.

Lipidomics analysis in plasma

Lipidomics analysis was carried out in 4-hour fasted plasma collected at week 6 of 8 mice per treatment group on the commercial Lipidyzer platform, according to the manufacturer's instructions (Sciex).

Excluded data

Of one mouse, a TC/non-HDL-C value misses (t=0 weeks) and of three mice, a HDL-C/non-HDL-C value misses (control t=0 weeks, 1 mouse; imatinib t=3 weeks, 1 mouse; ponatinib t=6 weeks, 1 mouse) as there was not enough plasma to measure TC and/or HDL-C. Six mice were excluded from analysis of the VLDL clearance experiment because the VLDL-like particles containing [3H]-TO and [14C]-CO, were not fully intravenously injected as confirmed by the absence of [14C]-CO in plasma collected at t=0 minutes (control, 1 mouse; nilotinib, 2 mice), or because the clearance curve showed aberrant results (i.e. higher [14C]-CO levels compared to the previous time point) (ponatinib, 3 mice). Four mice were excluded from the VLDL-TG production experiment: in two mice the [35S]-label was not fully intravenously injected demonstrated by absence of [35S]-decay in the plasma (control, 1 mouse; nilotinib, 1 mouse) and two mice were excluded as there was no plasma left to measure [35S]-decay (imatinib, 1 mouse; ponatinib, 1 mouse).

Data and statistical analysis

Data are presented as means ± SEM. A Kruskal–Wallis test was used to determine the significance of differences between the groups. Significance of differences of the individual groups with the control was calculated nonparametrically using a Mann–Whitney U-test. The lipidomics data were analyzed using a one-way ANOVA, and when the significance level of F was $P < 0.05$, a Dunnett's post hoc test was used to compare the treatment groups with the control group. IBM SPSS v24.0 was used for all analyses and P values < 0.05 were considered statistically significant. The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (31).

Results

Plasma drug concentrations

Based on data of our previously performed PK study (15) treatment with imatinib (150 mg/kg, BID), nilotinib (10 mg/kg, QD) and ponatinib (3 mg/kg, QD) resulted in similar drug exposures as reported in CML-patients (15).

Safety aspects of TKI treatment

No clinical signs of deviant behavior were noted in any treatment group. TKI treatment did not affect food intake or body weight in the mice (**Table 2**). Plasma ALT and AST, measured after 3 and 6 weeks of treatment, showed no aberrant results (**Table 2**). Mean body weight, food intake, ALT and AST were similar for mice of experiment 1 (VLDL clearance) and experiment 2 (VLDL production) for each treatment group (**Table 2**). Analogous to this short-term study, no adverse/toxic side-effects of the drugs were noted during long-term (16 weeks) exposure to the same doses for imatinib (150 mg/kg, BID), nilotinib (30 and 10 mg/kg, QD) and ponatinib (10 and 3 mg/kg, QD), as reported previously (15).

The effect of TKI treatment on plasma lipids

Imatinib and ponatinib decreased plasma cholesterol

The effect of TKI treatment on plasma lipids was assessed throughout the study (**Figure 1 and Table 2**). Imatinib markedly decreased plasma TC (**Figure 1A**), non-HDL-C (i.e. apoB-containing lipoproteins) (**Figure 1B**) and TG (**Figure 1C**) levels after 3 and 6 weeks of treatment, and ponatinib decreased plasma TC and non-HDL-C levels (**Figure 1A and C**). Nilotinib reduced plasma TG after 6 weeks of treatment (**Figure 1C**). These data confirm our previous findings (15) and correspond with the lipid-lowering properties of imatinib in patients (7–10).

Table 2 Overview of body weight, food intake and plasma parameters of mice in experiment 1 and 2

Experiment 1									
		Control		**Imatinib**		**Nilotinib**		**Ponatinib**	
Dose	*mg/kg*	-		150		30	10	10	3
Time	*weeks*	3	6	3	6	3	6	3	6
Body weight	*gram*	19.9±0.7	20.1±0.9	20.3±1.1	20.3±1.0	20.6±1.9	21.4±2.6	20.8±2.0	20.9±2.3
Food intake	*gram/mouse/day*	2.4±0.0	2.1±0.1	2.3±0.0	2.1±0.1	2.4±0.1	2.2±0.0	2.4±0.0	2.0±0.1
TC	*mmol/L*	17.6±2.2	13.3±2.8	7.2±1.9*	6.2±2.0*	15.9±4.1	11.3±3.1	10.5±1.8*	8.4±1.7*
HDL-C	*mmol/L*	1.0±0.4	0.8±0.2	0.6±0.2	0.5±0.1*	0.7±0.3	0.6±0.2	0.7±0.2	0.6±0.1
Non-HDLC	*mmol/L*	17.0±2.1	12.5±2.8	6.8±2.1*	5.7±2.1*	15.2±3.9	10.7±2.9	9.8±1.8*	7.7±1.8*
TG	*mmol/L*	4.6±1.3	4.3±1.5	1.5±0.6*	2.2±0.8*	4.4±1.2	2.5±1.1*	3.3±1.0	3.5±1.2
ALT	*U/L*	45	33	54	32	41	32	43	31
AST	*U/L*	109	88	98	79	128	72	145	82
Experiment 2									
		Control		**Imatinib**		**Nilotinib**		**Ponatinib**	
Dose	*mg/kg*	-		150		30	10	10	3
Time	*weeks*	3	6	3	6	3	6	3	6
Body weight	*gram*	20.3 ± 1.5	20.9±1.7	20.5±0.6	20.9±0.8	19.5±1.4	20.2±1.5	19.9±1.3	20.9±1.4
Food intake	*gram/mouse/day*	2.4±0.4	2.3±0.2	2.4±0.0	2.5±0.4	2.2±0.1	2.1±0.1	2.3±0.1	2.1±0.1
TC	*mmol/L*	14.6±4.4	11.9±4.3	8.3±2.7*	6.5±1.9*	13.0±4.3	10.9±2.2	8.7 ±1.8*	10.5±2.0
HDL-C	*mmol/L*	0.6 0.2	0.5±0.1	0.4±0.1*	0.5±0.2	0.6±0.3	0.6±0.2	0.8±0.5	0.6±0.3
Non-HDL-C	*mmol/L*	13.9±4.2	11.4±4.3	7.9±2.7*	6.1±1.9*	12.4±4.2	10.3±2.2	7.9±1.7*	9.9±2.0
TG	*mmol/L*	3.7±1.9	2.9±1.3	3.1±1.1	2.0±0.7	4.4±2.2	2.8±0.9	4.7±1.9	3.4±1.4
ALT	*U/L*	40	36	52	29	42	30	46	27
AST	*U/L*	138	120	119	96	119	93	135	113

Mice received a Western-type diet and were treated for 6 weeks with 3 generations TKIs, imatinib (150 mg/kg BID), nilotinib (30 mg/kg QD for the first 3 weeks and 10 mg/kg QD during the last 3 weeks) or ponatinib (10 mg/kg QD first 3 weeks and 3 mg/kg QD last 3 weeks). All parameters were measured after 3 and 6 weeks of treatment. Food intake was measured per cage and ALT and AST were measured in plasma pooled per group. After 6 weeks, two different endpoint experiments were performed. In Experiment 1 VLDL clearance was assessed, and experiment 2 determined VLDL production. (n=7-8 mice per experiment). *p<0.05 as compared to the control group. Abbreviations: TC, total cholesterol; HDL, high-density-lipoprotein; TG, triglycerides; ALT, alanine transaminase; AST, aspartate transaminase

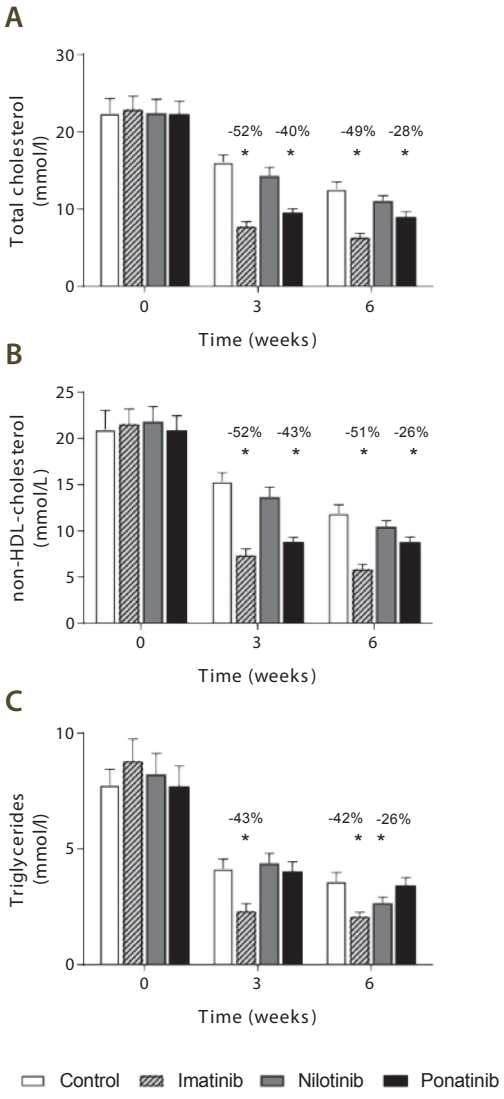

Figure 1 Imatinib and ponatinib decrease plasma lipids. Mice received a Western-type diet and were treated for 6 weeks with 3 generations TKIs, imatinib (150 mg/kg BID), nilotinib (30 mg/kg QD for the first 3 weeks and 10 mg/kg QD during the last 3 weeks) or ponatinib (10 mg/kg QD first 3 weeks and 3 mg/kg QD last 3 weeks). At baseline (t0) and after 3 and 6 weeks of intervention, 4-h fasted blood was taken and plasma was assayed for TC (A), non-HDL C (B) and TG (C). Non-HDL-C was calculated by subtracting HDL-C from TC. Data are presented as means + SEM. n=16-17 per group. *p<0.05 as compared to the control group. Abbreviations: TC, total cholesterol; (non)-HDL-C, high-density lipoprotein-cholesterol; TG, triglycerides

The effect of TKI treatment on lipoprotein and lipid metabolism

Imatinib decreased de novo VLDL synthesis and CE content of VLDL lipoproteins

Plasma VLDL levels are defined by the balance between VLDL production and VLDL clearance. We first determined the clearance and tissue uptake of glycerol tri[3H]oleate ([3H]-TO), representing FFAs and TGs, and [14C]cholesteryl oleate ([14C]-CO) by several organs at t=6 weeks in experiment 1. The clearance and tissue uptake of [3H]TO (**Figure 2A-B**) and of [14C]CO (**Figure 2C-D**) were not affected by TKI treatment, indicating that the lipid-modulating effects of imatinib and ponatinib cannot be explained by an increased VLDL clearance.

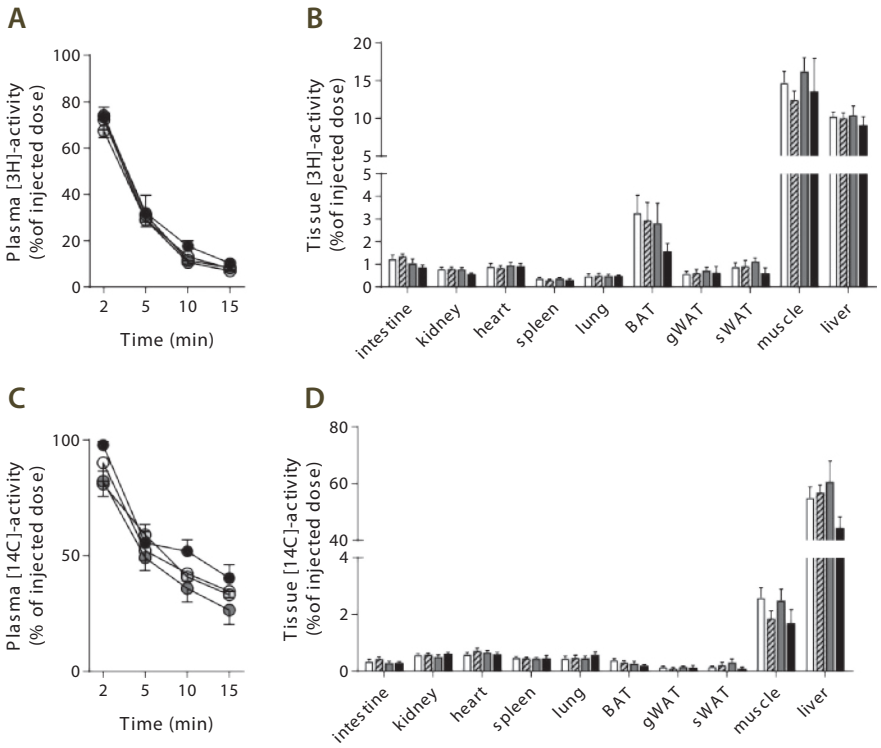

Figure 2 TKI treatment does not affect VLDL clearance and uptake. At t=6 weeks, 4-h fasted mice in experiment 1 were injected with glycerol tri[3H]oleate ([3H]TO) and [14C]cholesteryl oleate ([14C]CO)-labeled emulsion particles. [3H]TO plasma decay was plotted (A) and clearance of ([3H]TO) in individual organs was determined (B). [14C]CO plasma decay was plotted (C) and clearance of [14C]CO in individual organs was determined (D). Data are represented as mean ± SEM (n=5-8 per group). Abbreviations: Intestine, small intestine; BAT, brown adipose tissue; gWAT, gonadal white adipose tissue; sWAT, subcutaneous white adipose tissue; muscle, muscle femoralis

Next, we determined the VLDL-TG and VLDL-apoB production rate and the composition of the VLDL particles. Imatinib reduced VLDL-TG production (**Figure 3A-B**) and *de novo* VLDL-apoB synthesis (**Figure 3C**). Consequently, the TG production per apoB was not affected by imatinib (**Figure 3D**), indicating that the number of newly synthesized VLDL particles is decreased. Furthermore, imatinib and ponatinib decreased the amount of CE in the VLDL particles (**Figure 3E**). In contrast, nilotinib did not affect VLDL production. Altogether, these data demonstrate that imatinib reduced plasma TC and TG levels by decreasing the number of newly synthesized VLDL particles and the CE content of the VLDL particles, whereas ponatinib decreased the CE content of the particles.

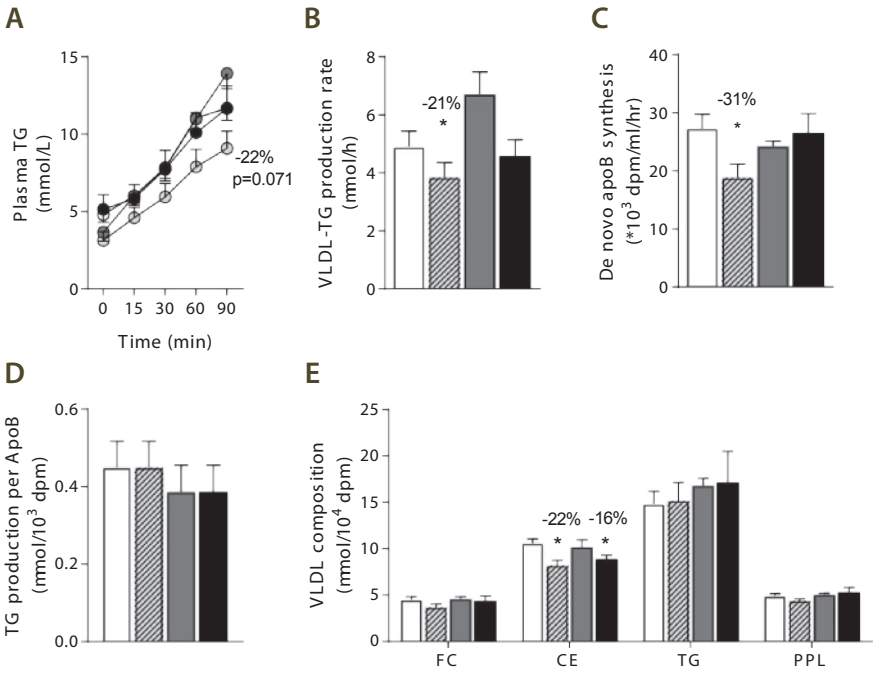

Figure 3 Imatinib decreases de novo VLDL-apoB production. At t=6 weeks, 4-h fasted mice in experiment 2 were injected with Tran[35S]-label and Triton after which blood samples were drawn up to 90 minutes. Plasma VLDL-TGs were plotted (A) and used to calculate the TG production rate (B) from the slope of individual curves. Ninety minutes after Triton injection plasma was used to isolate VLDL by ultracentrifugation, and the rate of de novo apoB synthesis was determined (C). Next, we calculated the TG production per apoB (D) and determined the lipid composition of the isolated VLDL particles (E). Data are represented as mean ± SEM (n=7-9 per group). *p<0.05 as compared to the control group. Abbreviations: FC, free cholesterol; CE, cholesterol ester; TG, triglycerides; PPL, phospholipids; apoB, apolipoprotein B.

Ponatinib decreased hepatic CE content and cholesterol absorption

Because a decreased VLDL-apoB particle production rate may be the result of changes in hepatic lipid metabolism, we determined hepatic lipid content and fecal excretion of bile acids and neutral sterols.

Imatinib did not affect hepatic lipid content (**Figure 4A**), indicating that the reduced VLDL particle production does not result in hepatic lipid accumulation, nor is the consequence of reduced availability of lipids for VLDL synthesis. Interestingly, ponatinib decreased hepatic CE content (**Figure 4A**), which may be related to reduced intestinal cholesterol absorption (30,32). Therefore, we measured fecal neutral sterol excretion and

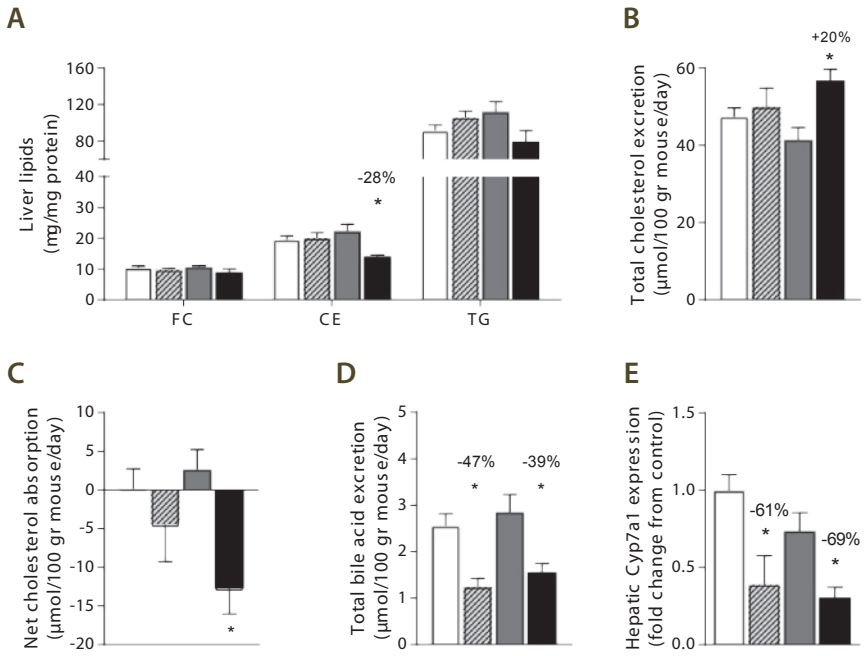

Figure 4 Ponatinib decreases hepatic CE content, bile acid excretion and cholesterol absorption. At t=6 weeks, livers were isolated from mice in experiment 1 and hepatic lipid content per mg liver protein was assessed (A). At t=5 weeks, feces from both experiments were collected per cage during a 48-72h period at 3 consecutive time points, in which neutral sterol excretion (B) was determined using gas chromatography. Total cholesterol balance (C) was calculated by subtracting fecal cholesterol excretion from dietary cholesterol intake. Bile acids (D) were determined in feces using gas chromatography, and Cyp7a1 mRNA expression was measured in the liver (E). Data are represented as mean + SEM (n=8 mice per group; n=4 cages and 3 time points per group). *p<0.05 as compared to the control group. Abbreviations: FC, free cholesterol; CE, cholesterol ester; TG, triglycerides.

calculated the net cholesterol absorption by subtracting the fecal neutral sterol excretion from the average dietary cholesterol intake (**Figure 4C**). Indeed, ponatinib increased the total cholesterol excretion (**Figure 4B**), thereby decreasing the net cholesterol absorption by 12.7 µmol/100 gram mouse per day (**Figure 4C**). Imatinib and nilotinib did not affect intestinal cholesterol absorption (**Figure 4C**). In addition, both imatinib and ponatinib reduced bile acid excretion (**Figure 4D**), via downregulation of the expression of *Cholesterol 7alpha-hydroxylase* (*Cyp7a1*), encoding the rate-limiting enzyme in bile acid synthesis (**Figure 4E**). Altogether, these data indicate that ponatinib reduced intestinal cholesterol uptake and consequently hepatic CE content, whereas imatinib did not affect hepatic lipid storage.

Plasma lipidomics analysis

Imatinib reduced the majority of lipid classes in plasma

Bioactive lipids, in particular sphingolipids including ceramides, are major regulators of lipid homeostasis (33) and together with (lyso-)glycerophospholipids play a role in atherogenesis (34–36). Therefore, we performed lipidomic analyses of plasma samples to identify characteristic molecular lipid changes of TKI treatment. Concentrations of almost all lipid classes were reduced by imatinib (**Figure 5A**), and the reductions in CE (-62%) and triacylglycerols (TAG) (-60%) were comparable with the observed reductions in plasma cholesterol (-49%) and TG (-42%) at 6 weeks of treatment (**Figure 1A and C**). These decreases were accompanied by reductions in the concentrations of glycerophospholipids (phosphatidylcholines and phosphatidylethanolamines), a subgroup of lipids that form the outer layer of lipoproteins, but are also recognized for their role in atherosclerosis development (36). Moreover, imatinib reduced the concentration of the pro-atherogenic sphingomyelins (-49%), ceramides (-55%), hexosylceramides (-50%) and lysophosphatidylcholines (LPC) (-45%) (**Figure 5A**). In contrast, ponatinib only reduced the concentration of CE by 38% (**Figure 5C**), in line with the decrease in plasma TC, without affecting TAG, phospholipids or sphingolipids. Nilotinib did not affect any of the lipid classes (**Figure 5B**). Normalization for plasma non-HDL-C levels revealed that the majority of the observed changes was associated with plasma non-HDL-C levels (**Figure 5 D-F**), except for the concentration of FFA in imatinib-treated mice, which was increased by 100% per µM non-HDL-C (**Figure 5D**) as expected, since FFA is bound to albumin and not present as such in lipoproteins. Collectively, the combined reduction in CE, TAG and phospholipids by imatinib confirms our finding that the drug decreases the number of VLDL particles, while the decreased concentration of sphingomyelins is in accordance with a more favorable cardiovascular profile of imatinib. The absolute concentrations for each lipid class quantified in mouse plasma are presented in **Table 3**.

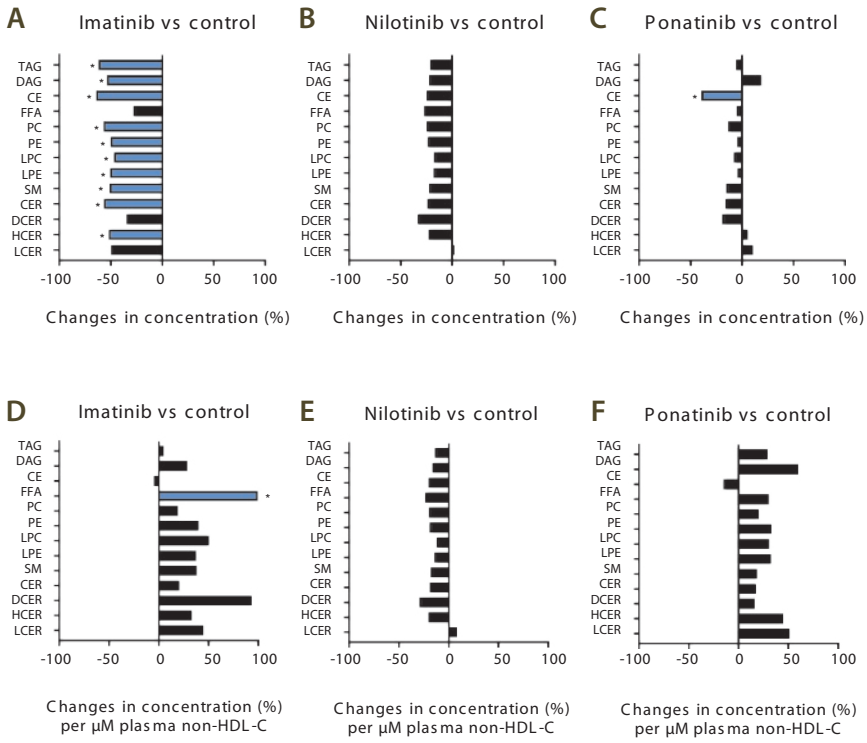

Figure 5 Differences in lipid class concentrations in plasma of mice treated with imatinib and ponatinib. After 6 weeks of treatment, 4-h fasted blood was taken, lipids were extracted from the plasma and the concentration (nmol/gram plasma) of 13 lipid classes was determined by MS/MS. The mean changes in concentration (%) for each lipid class compared to the control group are depicted for imatinib (A), nilotinib (B) or ponatinib (C). Next, the mean change in concentration (%) per µM plasma non-HDL cholesterol was calculated for each lipid class compared to the control group and depicted for imatinib (D), nilotinib (E) and ponatinib (F). Significance of differences between the treatment groups versus control was calculated using an one-way ANOVA with Dunnett post-hoc test using the data of which the means are depicted in **Table 3**. (n=8 mice per group). *p<0.05 as compared to the control group with significant changes shown in blue bars. Abbreviations: Non-HDL-C, non-high density-lipoprotein cholesterol; TAG, triacylglycerols; DAG, diacylglycerols; CE, cholesteryl esters; FFA, free fatty acids; PC, phosphatidylcholines; PE, phosphatidylethanolamines; LPC, lyso-phosphatidylcholines; LPE, lyso-phosphatidylethanolamines; SM, sphingomyelins; CER, ceramides; DCER, dihydroceramides; HCER, hexosylceramides; LCER, lactosylceramides.

Table 3 Plasma concentrations of lipid classes

	Concentration (nmol/gram plasma)			
Lipid class	**Control**	**Imatinib**	**Nilotinib**	**Ponatinib**
TAG	3697 ± 1798	1478 ± 901*	2979 ± 825	3551 ± 1926
DAG	52.6 ± 21.0	25.3 ± 13.3*	41.7 ± 5.9	62.4 ± 24.5
CE	10905 ± 4036	4092 ± 1012*	8407 ± 2383	6800 ± 2435*
FFA	1352 ± 474	1001 ± 151	1008 ± 177	1309 ± 388
PC	3854 ± 1237	1724 ± 503*	2973 ± 551	3411 ± 1246
PE	117.6 ± 39.2	60.8 ± 15.4*	91.8 ± 13.7	114.8 ± 46.6
LPC	1035 ± 345	571 ± 134*	871 ± 162	974 ± 299
LPE	13.3 ± 5.1	6.8 ± 1.7*	11.2 ± 1.9	13.0 ± 5.1
SM	1086 ± 385	550 ± 164*	862 ± 171	940 ± 345
CER	19.1 ± 7.4	8.6 ± 3.3*	14.9 ± 2.6	16.4 ± 6.0
DCER	1.9 ± 0.8	1.3 ± 0.7	1.3 ± 0.3	1.5 ± 0.4
HCER	18.9 ± 8.3	9.4 ± 2.6*	14.9 ± 3.7	19.9 ± 7.0
LCER	1.1 ± 0.7	0.6 ± 0.3	1.1 ± 0.2	1.2 ± 0.5

After 6 weeks of treatment lipidomic analysis was performed in 4-hour fasted blood (n=8 mice per group). *p<0.05 as compared to the control group. Abbreviations: TAG, triacylglycerols; DAG, diacylglycerols; CE, cholesteryl esters; FFA, free fatty acids; PC, phosphatidylcholines; PE, phosphatidylethanolamines; LPC, lyso-phosphatidylcholines; LPE, lyso-phosphatidylethanolamines; SM, sphingomyelins; CER, ceramides; DCER, dihydroceramides; HCER, hexosylceramides; LCER, lactosylceramides.

Discussion

To our knowledge, this is the first *in vivo* study that investigated the effects of three different BCR-ABL1 tyrosine kinase inhibitors on lipoprotein metabolism, of which the results are summarized in **Table 4**. We confirm the lipid-lowering ability of imatinib as reported in CML patients, and provide evidence using APOE*3-Leiden.CETP mice that this is caused by a reduction of the VLDL particle production and CE content of the VLDL particles. This observation is supported by lipidomics analysis showing a reduction of glycerophospholipids in the plasma, which form the outer layer of the VLDL particles. The decreased concentration of the pro-atherogenic sphingomyelins, ceramides and (lyso-)phospholipids further contributes to the favorable cardiovascular safety profile of imatinib (11,15). Ponatinib also reduced plasma cholesterol, but the identified mechanism of action differed from imatinib. A decrease of intestinal cholesterol absorption led to a reduction of hepatic CE content, an accumulative process as long-term (16-week) exposure to ponatinib further decreased hepatic lipid content, including FC and TG (15). As a consequence

Table 4 Summary of the (cardio)metabolic effects of imatinib, nilotinib and ponatinib in APOE*3-Leiden.CETP mice

	Effects in APOE*3-Leiden.CETP mice		
	Imatinib	**Nilotinib**	**Ponatinib**
Plasma parameters			
Total cholesterol	↓		↓
Non-HDL cholesterol	↓		↓
Triglycerides	↓	↓*1	
Pro-atherogenic sphingolipids	↓		
Lipoprotein metabolism			
VLDL particle production rate	↓		
CE content of the VLDL particles	↓		↓
Hepatic lipid content			↓
Intestinal lipid absorption			↓
Cardiovascular safety effects*2			
Atherosclerosis progression	↓		↓
Coagulation		↑	↑

Mice received a Western-type diet and were treated for 6 weeks with 3 generations TKIs, imatinib (150 mg/kg BID), nilotinib (30 mg/kg QD for the first 3 weeks and 10 mg/kg QD during the last 3 weeks) or ponatinib (10 mg/kg QD first 3 weeks and 3 mg/kg QD last 3 weeks). Plasma parameters were measured after 3 and 6 weeks of treatment. Intestinal lipid absorption was measured after 5 weeks of treatment, and the parameters regarding lipoprotein metabolism were measured at end-point. *1 reduced at t=6 weeks, not at t=3 weeks. *2 The effects on atherosclerosis and coagulation were determined in our previous study (15).

of the limited substrate availability for lipoprotein synthesis, the CE content of the VLDL particles was reduced. In contrast to imatinib, the VLDL-TG production rate was maintained and thus, plasma TG levels were not affected. Ponatinib did not additionally reduce sphingolipids, which contributes to the pro-atherogenic profile of the drug (2,3). Importantly, nilotinib had no effect on lipoprotein metabolism and it is reasonable to believe that the lipid elevations reported with nilotinib in CML-patients (1,10,12,13,37) are caused by response to treatment rather than being a direct effect of off-target (kinase) inhibition.

In CML-patients, it is challenging to distinguish between disease-related lipid modulation or off-target effects of TKI treatment on lipoprotein metabolism. Reduced caloric intake and increased energy requirements imposed by tumor growth may result in reduced plasma cholesterol and TG levels at the moment of diagnosis (38), whereas good response to treatment increases plasma lipid levels again (39,40). In addition, early versus late diagnosis, individual patient characteristics, the presence of one or more risk factors, and previous or current treatment with other TKIs or drugs may affect plasma cholesterol

levels. To exclusively focus on the off-target effects of TKI-treatment, we have used a mouse model without CML, but with a human-like lipoprotein metabolism, the APOE*3-Leiden.CETP mouse model. Unlike wildtype rodents, these mice have a delayed apoE-LDLR-mediated clearance pathway of atherogenic apoB-containing lipoproteins and express CETP (16), and develop hypercholesterolemia upon a Western-type diet. This well-characterized model has been widely used to study lipoprotein metabolism (22,24–26,41) and responds similarly as humans do to hypolipidemic drugs used in the clinic, including statins, fibrates, niacin and PCSK9-inhibitors (18,19,25,42,43). To further improve the translatability of our results, the dose and dose interval of the treatments were based on data of our previously performed PK study (15), resulting in drug concentrations similar as in CML-patients. The lipid-lowering effects of imatinib and ponatinib were induced within 3 weeks and the magnitude of the changes in plasma lipid levels were consistent with our previous observations in APOE*3-Leiden.CETP mice (15). Moreover, the lipid-lowering properties of imatinib are in line with findings in CML-patients (7–10).

Cardiovascular safe profile of imatinib

Our previous study suggested that the lower incidence of cardiovascular events in patients treated with imatinib (11) are the result of reduced plasma lipids and atherosclerosis progression without adverse pro-thrombotic effects as observed with other TKIs (15). In this study, we provide evidence that this is the result of reduced VLDL particle synthesis, illustrated by the decreased (I) VLDL-TG and VLDL-apoB production, (II) CE content of the VLDL particles, and (III) the concentration plasma glycerophospholipids. In line with our findings, *de novo* fatty acid synthesis is reduced when myeloid tumor cells are treated with imatinib (44), pointing towards decreased VLDL particle production.

Parallel to the lipid-modulating effects, imatinib decreased the concentration of pro-atherogenic sphingolipids, amphiphilic molecules that are associated with lipoproteins, of which VLDL and LDL contain most of the sphingomyelins and ceramides (34). Relevant pro-atherogenic processes that are promoted by sphingomyelins and ceramides include lipoprotein aggregation, induction of macrophage foam cell formation, plaque instability and stimulation of pro-inflammatory responses (reviewed in (34,45)). Moreover, imatinib reduced the concentration LPCs, which increase endothelial inflammation (36) and activate signal-transduction cascades involved in the initiation and progression of atherosclerosis (46). Plasma concentrations of ceramides and sphingomyelins are elevated in patients with unstable angina pectoris and acute myocardial infarction (47), specific ceramides are associated with plaque instability and cardiovascular death (48,49), and some LPC species are identified as diagnostic markers for myocardial infarction (50), indicating that the lipid-associated reduction in sphingolipids as observed in our study is relevant for CML-patients treated with imatinib. The reduction of plasma TC, TG and pro-atherogenic sphingolipids may all have contributed to the reduced atherosclerosis development (15) and the favorable cardiac profile in CML-patients (11).

Cardiovascular toxic effects of nilotinib and ponatinib

In contrast to the cardiovascular safe profile of imatinib, the second and third line BCR-ABL1 tyrosine kinase inhibitors nilotinib and ponatinib increase the onset of arterial occlusive events (1–4,12), especially in those patients having a high or very high cardiovascular risk according to the SCORE chart that includes sex, age, smoking habits, systolic blood pressure and TC levels (5,6). Nilotinib did not directly affect lipoprotein metabolism and the lipid elevations reported in nilotinib-treated CML-patients are probably related to response to treatment (39,40). Nevertheless, these elevations may add to the cardiovascular risk (reviewed in (2)), and it is therefore recommended to monitor plasma cholesterol levels when subscribing nilotinib.

Interestingly, ponatinib decreased plasma TC levels by reducing intestinal cholesterol absorption and we previously reported that such a decrease was correlated to reduced atherosclerosis progression (15). Despite, these favorable processes are probably dominated by several negative effects. The decrease in plasma cholesterol and subsequently reduced atherosclerosis development in APOE*3-Leiden.CETP mice was less pronounced with ponatinib as compared to imatinib (15), and therefore might be abolished by response to treatment in patients. In addition, whereas imatinib reduced the concentration of pro-atherogenic glycerophospholipids in the plasma, this was not observed with ponatinib. More importantly, however, we and others have shown that both nilotinib and ponatinib increase blood coagulability (15,51–54), essential in the pathogenesis of atherothrombotic events. These findings, together with the observation of the strongly increased risk in patients predisposed with adverse traditional cardiovascular risk factors (2,5,6) underline the importance to carefully monitor patients during nilotinib and ponatinib treatment.

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