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Effects of mineral oil administration on the pharmacokinetics, metabolism and pharmacodynamics of atorvastatin and pravastatin in mice and dogs

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ABSTRACT

We investigated the effects of mineral oil on statin pharmacokinetics and inflammatory markers in animal models. A new synthesis strategy produced regioisomers that facilitated the characterization of the main metabolite (M1) of atorvastatin, a lipophilic statin, in C57BL/6NCrl mice. The chemical structure of M1 in mice was confirmed as ortho-hydroxy β-oxidized atorvastatin. Atorvastatin and M1 pharmacokinetics and inflammatory markers were assessed in C57BL6/J mice given atorvastatin 5 mg/kg/day or 10 mg/kg/day, as a single dose or for 21 days, with or without 10 μL or 30 μL mineral oil. No consistent differences in plasma exposure of atorvastatin or M1 were observed in mice after single or repeat dosing of atorvastatin with or without mineral oil. However, mice administered atorvastatin 10 mg/kg with 30 μL mineral oil for 21 days had significantly increased plasma levels of serum amyloid A (mean 9.6 μg/mL vs 7.9 μg/mL without mineral oil; $p < 0.01$) and significantly increased proportions of $C62L^{high}$ B cells (mean 18% vs 12% without mineral oil; $p = 0.04$). There were no statistically significant differences for other inflammatory markers assessed. In dogs, pharmacokinetics of atorvastatin, its two hydroxy metabolites and pravastatin (a hydrophilic statin) were evaluated after single administration of atorvastatin 10 mg plus pravastatin 40 mg with or without 2 g mineral oil. Pharmacokinetics of atorvastatin, hydroxylated atorvastatin metabolites or pravastatin were not significantly different after single dosing with or without mineral oil in dogs. Collectively, the results in mice and dogs indicate that mineral oil does not affect atorvastatin or pravastatin pharmacokinetics, but could cause low-grade inflammation with chronic oral administration, which warrants further investigation.

Introduction

Recent studies assessing the effects of omega-3 fatty acids on triglyceride levels and cardiovascular risk typically use oil-based placebo capsules such as mineral oil as the control. Such placebo administrations are assumed to be inert and to have no impact on efficacy and safety read-outs in the clinical trials. There is, however, limited published information on the biological effects of orally administered mineral oil (European Food Safety Authority (EFSA) Panel on Contaminants in the Food Chain (CONTAM), 2012). Mineral oil consists of a mixture of hydrocarbons and, although its oral absorption is very low, the European Food Safety Authority concluded in 2012 that mineral oil may undergo some gastrointestinal absorption and tissue accumulation in a dose- and time-dependent manner; the level of absorption varies depending on the

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mineral oil hydrocarbon structure and molecular mass/carbon chain length (European Food Safety Authority (EFSA) Panel on Contaminants in the Food Chain (CONTAM), 2012). A recent overview based on current European regulations similarly concluded that mineral oil is differentially absorbed in the intestine depending on carbon chain length, and can accumulate in tissues due to low biotransformation rate (Chuberre et al., 2019). By increasing gastrointestinal transit and/or creating a lipid barrier lining of the intestinal wall, mineral oil can also affect drug absorption in the gastrointestinal tract (Prescriber's Digital Reference, 2021; Steigmann et al., 1952). The absorption of fat soluble vitamins, for example, is reduced in the presence of mineral oil (Prescriber's Digital Reference, 2021; Steigmann et al., 1952). Lipophilic statins, which have been reported to have good solubility in mineral oil (Mustafa et al., 2009; Ho and Walker, 2012), may also experience reduced gastrointestinal absorption when mineral oil is present.

Results from omega-3 fatty acids trials that used mineral oil as a placebo showed an increase in the mineral oil placebo groups for levels of non-high-density lipoprotein (non-HDL) cholesterol, C-reactive protein and apolipoprotein B (Bays et al., 2011; Ballantyne et al., 2012; Bhatt et al., 2019), which are markers for inflammation and coronary artery disease risk (Ridker et al., 1997; Ridker et al., 2000; Ference et al., 2017), leading to concerns over whether the use of mineral oil as a placebo exaggerates any benefits seen in the treatment arm (FDA Briefing Document, 2019; Haslam and Prasad, 2019; Kastelein and Stroes, 2019). Knowing whether mineral oil has biological activity is important for the interpretation of results from clinical trials using it as a placebo – which impacts recommendations for clinical practice – and for the design of future placebo-controlled omega-3 fatty acid trials. In addition, in accordance with regulatory agreement with its use as a placebo (FDA Briefing Document, 2019), mineral oil can be used in drug formulation, and any potential activity may thus also have implications for pharmaceutical product manufacturing.

Our aim was to obtain a better understanding of the potential effect of mineral oil as a placebo or as a formulation in clinical trials. For the animal studies reported here, we had two hypotheses: mineral oil would lower the intestinal absorption of statins, thereby decreasing their bioavailability and beneficial effects; and/or chronic use of mineral oil would cause the intestine to become irritated, leading to low-grade inflammation and increased cardiovascular risk. Atorvastatin, one of the most commonly prescribed statins, is lipophilic and has been reported to have good solubility in mineral oil (Mustafa et al., 2009; Ho and Walker, 2012), thus its gastrointestinal absorption and clinical efficacy may be reduced in the presence of mineral oil in the gut. We added a hydrophilic statin, pravastatin (Ho and Walker, 2012), to elucidate any relationship between Biopharmaceutics Classification System (BCS) characteristics and interaction with mineral oil. In this work, we investigated the hypotheses as follows: (1) assessed the effect of mineral oil on the pharmacokinetics of atorvastatin and its newly characterized main metabolite in mice, after single and repeated daily oral dosing; (2) measured the effect of mineral oil on inflammatory markers in plasma, small intestine and colon in mice after repeated oral dosing; (3) assessed the effect of mineral oil on the pharmacokinetics of atorvastatin, including its main active hydroxy metabolites, as well as pravastatin in Labrador dogs (a second, non-rodent pharmacological preclinical species) after single dosing.

Material and methods

Materials

Chemicals and formulations

Mineral oil light, consisting of a mixture of paraffinic hydrocarbons (aliphatic hydrocarbons, viscosity 15.3 centistokes at 40°C), used for the studies in mice and in dogs was purchased from Sigma-Aldrich (USA). For the bioanalysis, [$^2H_5$]-atorvastatin, [$^2H_5$]-*o*- and [$^2H_5$]-*p*-hydroxy atorvastatin, and [$^2H_9$]-pravastatin were purchased from AlsaChim (France), and 5,5-diethyl-1,3-diphenyl-2-iminobarbituric acid was purchased from Sigma-Aldrich (USA).

For the studies in mice, atorvastatin calcium salt was purchased from eNovation Chemicals LLC. The metabolites ortho (*o*)-hydroxy β-oxidized atorvastatin (M1), and para (*p*)- and meta (*m*)-hydroxy β-oxidized atorvastatin were synthesized under a service agreement contract by Pharmaron (Beijing, China) using a synthetic protocol designed by AstraZeneca. For mice, atorvastatin stock suspension 20 mg/mL in 0.5% w/w hydroxypropyl methylcellulose (HPMC) 10000 centipoises (cps) (Shin-Etsu, Japan) + 0.1% w/w Polysorbate 80 (Seppic, France) in water and vehicle 0.5% w/w HPMC 10000 cps + 0.1% w/w Polysorbate 80 in water were prepared at AstraZeneca. Because the minimum volume that could be administered accurately by oral gavage to mice was 20 μL, for mineral oil doses below 20 μL a 60 μL/mL mineral oil-in-water emulsion was prepared, consisting of 60 μL/mL mineral oil in 0.5% w/w Span 80 (Sigma-Aldrich, Germany) + 0.5% w/w Polysorbate 80 in water.

For the study in dogs, Atorvastatin 10 mg (Krka dd Novo mesto, Novo Mesto, Slovenia) and Pravastatin 40 mg (Teva Pharmaceutical Works, Debrecen, Hungary) commercial tablets were purchased from a local pharmacy in Gothenburg, Sweden. Mineral oil light and alpha-tocopherol antioxidant (purchased from Sigma-Aldrich, USA, and Sigma-Aldrich, Germany, respectively) were filled into hard gelatine capsules (size 000, Fisher Scientific) at AstraZeneca. Each capsule contained 932 mg mineral oil and 1.86 mg antioxidant.

Other chemicals and solvents

Methanol and acetonitrile (liquid chromatography–mass spectrometry [LC–MS] grade) were obtained from Fisher Scientific (Loughborough, UK). Formic acid (98–100%) was obtained from Merck KGaA (Darmstadt, Germany). Ultra-pure water was prepared by an in-house water purification system (Milli-Q, Integral 3, Millipore Co.). All other chemicals and solvents were of the highest quality commercially available.

Animals

The preclinical *in vivo* studies in mice described herein were conducted according to protocols approved by the institutional animal care and use committees at AstraZeneca and TNO. The welfare of the mice was maintained in accordance with the general principles governing the use of animals in experiments of the European Communities (2010/63/EU) and the Dutch legislation (The Experiments on Animals Act, 2014). The *in vivo* dog study was performed at AstraZeneca Gothenburg and was approved by the local ethics committee for animal research (no: 34−2015) in Gothenburg, Sweden.

All mice were obtained from Charles River Laboratories (Germany). The atorvastatin major metabolite profile was characterized using female C57BL/6NCrl mice (10–12 weeks old, about 20 g body weight). Pharmacokinetic and pharmacodynamic parameters in mice were assessed using female specific pathogen-free (SPF) C57BL6/J mice (10–12 weeks old, about 20 g body weight). Mice were housed in communal cages kept in conventional animal rooms at approximately 21°C with a 12 hours light/12 hours dark cycle, and provided access to food and water *ad libitum*. The dog pharmacokinetic study was conducted using four male Labrador dogs 6−8 years of age and weighing 36−39 kg. The dogs were housed in pairs with free access to outdoor yard space during the daytime and weekly time in an outdoor agility course. They were provided access to water *ad libitum* up until study drug administration and from 3 hours post administration. Dogs were fed once daily with commercial dog ration.

General quantitative bioanalysis

The general approach for the LC–tandem MS (LC–MS/MS) bioanalysis of atorvastatin and its metabolites varied to some degree

according to the slightly different assay conditions used for the studies in mice and dogs. Pravastatin was analysed in dogs only.

In general, for sample preparation prior to bioanalysis, 20–50 μL of plasma was protein-precipitated with 150–200 μL of organic solvent consisting primarily of acetonitrile with internal standard. In the mouse study, metabolite M1 was semi-quantified against atorvastatin calibration standards because access to a synthesized standard was possible only at subsequent stages of this study. In a separate study, a comparison between M1 concentrations using either atorvastatin or M1 for calibration was subsequently performed with the same analytical method. The assay for the analysis of atorvastatin and its metabolites in the dog was conducted at Charles River Laboratories under a service contract agreement, using a validated methodology that prevents conversion of hydroxy atorvastatin to lactone (Jemal et al., 1999).

Methodological details, including the type of column, flow rate, gradient profile and analysis time, are summarized in Supplementary data Table S1. All samples were analysed using an Acquity ultra-high-performance liquid chromatography (UPLC) system coupled with a mass spectrometer from Waters, UK, except for atorvastatin in dog samples, which were analysed using a Sciex API 5000 from Sciex, USA. Both systems were operated in positive ionization mode for analysis of atorvastatin; pravastatin was analysed in negative ionization mode. Data analysis used linear regression with $1/X^2$ weighting.

Overall pharmacokinetic analysis

Non-compartmental pharmacokinetic analysis was performed with Phoenix WinNonlin version 8.1 (Certara, Princeton, USA), using the linear up logarithmic down trapezoidal method. The plasma parameters assessed for atorvastatin, its metabolites and pravastatin included maximum (peak) plasma concentration (C_{max}), time to reach maximum plasma concentration (t_{max}), area under the plasma concentration–time curve (AUC) from zero to infinity (AUC_{0-inf}), AUC from zero to time t (AUC_{0-t}) and terminal half-life ($t_{1/2}$). C_{max} and AUC ratios were calculated by dividing the values obtained with mineral oil by values obtained without mineral oil.

Metabolite characterization in mice

Experimental design

Four mice (strain C57BL/6NCrl) were used for the characterization of the atorvastatin major metabolite profile. On the day of dosing, mice were moved to single cages and received a single oral dose of atorvastatin (10 mg/kg) in the morning, after 2 hours of fasting. Blood samples for metabolite identification and characterization, and bioanalysis were collected via the saphenous vein at 1, 2 and 5 hours after dosing.

Chemical synthesis of hydroxy β-oxidized atorvastatin

Regioisomers of hydroxy β-oxidized atorvastatin were synthesized to facilitate the characterization of metabolite M1 in plasma from mice. To overcome the challenges associated with hydroxylation of the anilines at a final step in the synthetic sequence, a unique pathway was designed that involved initial insertion of *p*-, *m*- and *o*-benzyl-protected hydroxy anilines to arrive at *p*-, *m*- and *o*-hydroxy β-oxidized atorvastatin, respectively, without compromising the structural integrity of the desired metabolites. Supplementary data Figure S1 shows the general synthetic strategy for the desired hydroxy β-oxidized atorvastatin regioisomers.

Sample analysis for metabolite identification

Mouse plasma samples for metabolite identification were prepared using protein precipitation. To 50 μL of the plasma, 150 μL of refrigerated mixture of acetonitrile:methanol (v/v, 1:1) was added. The samples were vortex mixed for 1 minute, followed by centrifugation at 10 000 *g* and 4°C for 10 minutes. The resulting supernatant was separated and diluted by two volumes of water before analysis by UPLC–high-resolution mass spectrometry (HRMS). Supernatants from spiked blank plasma with reference standards were also prepared, to identify and confirm the structure of the main atorvastatin metabolite in mice. Sample separation and metabolite identification were performed using an Acquity UPLC system coupled with a Xevo G2-S QTOF mass spectrometer (Waters, UK) via an electrospray ionization interface (analytical column: Acquity BEH C18 2.1 × 100 mm, 1.7 μm; column temperature: 45°C; mobile phase A: 0.1% formic acid in water, mobile phase B: acetonitrile [gradient: from 25% B to 90% B over 6 minutes]; flow rate: 0.5 mL/minute; injection volume: 2 μL, MS acquisition range: *m/z* 80–1200, positive mode).

Metabolite structures were assigned based on: their HRMS full scan and MS/MS product mass spectra; the UPLC chromatographic retention times; and comparison with the UPLC–HRMS data obtained from the reference standards of atorvastatin and its synthesized hydroxy β-oxidized metabolites.

Pharmacokinetics and pharmacodynamics in mice

Experimental design

In a separate study, 70 mice (strain C57BL6/J) were used for the pharmacokinetic and pharmacodynamic studies. Mice were fed a semi-synthetic, Western-type diet containing 0.15% cholesterol, 15% cacao butter, 1% corn oil, 40.5% sucrose, 10% starch, 20% casein, 5.95% cellulose, 2% choline, 0.2% methionine, and 5.35% vitamin and mineral mix (all w/w) (Ssniff, Soest, Germany). After matching by body weight, mice were divided into seven groups of 10 animals each. The sample size of 10 mice per group was selected to provide 80% power to detect a between-group difference of 25%, based on an assumed coefficient of variation (CV) of 20%, using a two-tailed test with a 95% confidence interval (CI).

For atorvastatin single dosing, five groups of mice were first acclimatized to daily oral gavage with water (Groups 1 and 4) or mineral oil (Groups 2, 3 and 5) for 2 weeks. On the day of the experiment, the mice were fasted for 2 hours and subsequently received a single dose of atorvastatin by oral gavage, with a mineral oil or water control, as follows: Group 1: atorvastatin 5 mg/kg body weight + 170 μL water; Group 2: atorvastatin 5 mg/kg body weight + 10 μL mineral oil, administered as 170 μL of a 60 μL/mL mineral oil-in-water emulsion; Group 3: atorvastatin 5 mg/kg body weight + 30 μL mineral oil; Group 4: atorvastatin 10 mg/kg body weight + 30 μL water; Group 5: atorvastatin 10 mg/kg body weight + 30 μL mineral oil. Blood samples were collected via tail vein bleedings at 0.5, 1.5, 4, 8 and 24 hours (five animals per group) or at 0.25, 1, 2.5, 6 and 10 hours (the remaining five animals per group) after dosing. Mice were dosed in the morning.

For atorvastatin repeat once-daily dosing, two groups of mice received a daily dose of atorvastatin 10 mg/kg body weight in the morning by oral gavage for 22 days, administered with 30 μL H_2O (control; Group 6) or 30 μL mineral oil (Group 7). Mice were fasted for 2 hours before dosing on days 1 and 22. Mice were dosed in the mornings. The chronically dosed mice did not undergo prior acclimatization for oral gavage. Blood samples were collected via tail vein bleedings at a single time point on days –1, 1 (1 hour after dosing) and 10, and at multiple time points on day 22 (at 0.5, 1.5, 4, 8 and 24 hours [five animals per group] or at 0.25, 1, 2.5, 6 and 10 hours [the remaining five animals per group]).

All mice were euthanized by CO_2 inhalation following the last blood sample collection. Blood for fluorescence-activated cell sorting (FACS) analysis was collected by heart puncture. Small intestine and colon tissues from the chronic dose groups were prepared using the "Swiss-roll" method (Moolenbeek and Ruitenberg, 1981), formalin-fixed for 24 hours and embedded in paraffin.

Sample processing for pharmacokinetic and pharmacodynamic analysis

Plasma concentrations of atorvastatin and its main metabolite were measured after single dosing on day 1 and after repeat once-daily dosing

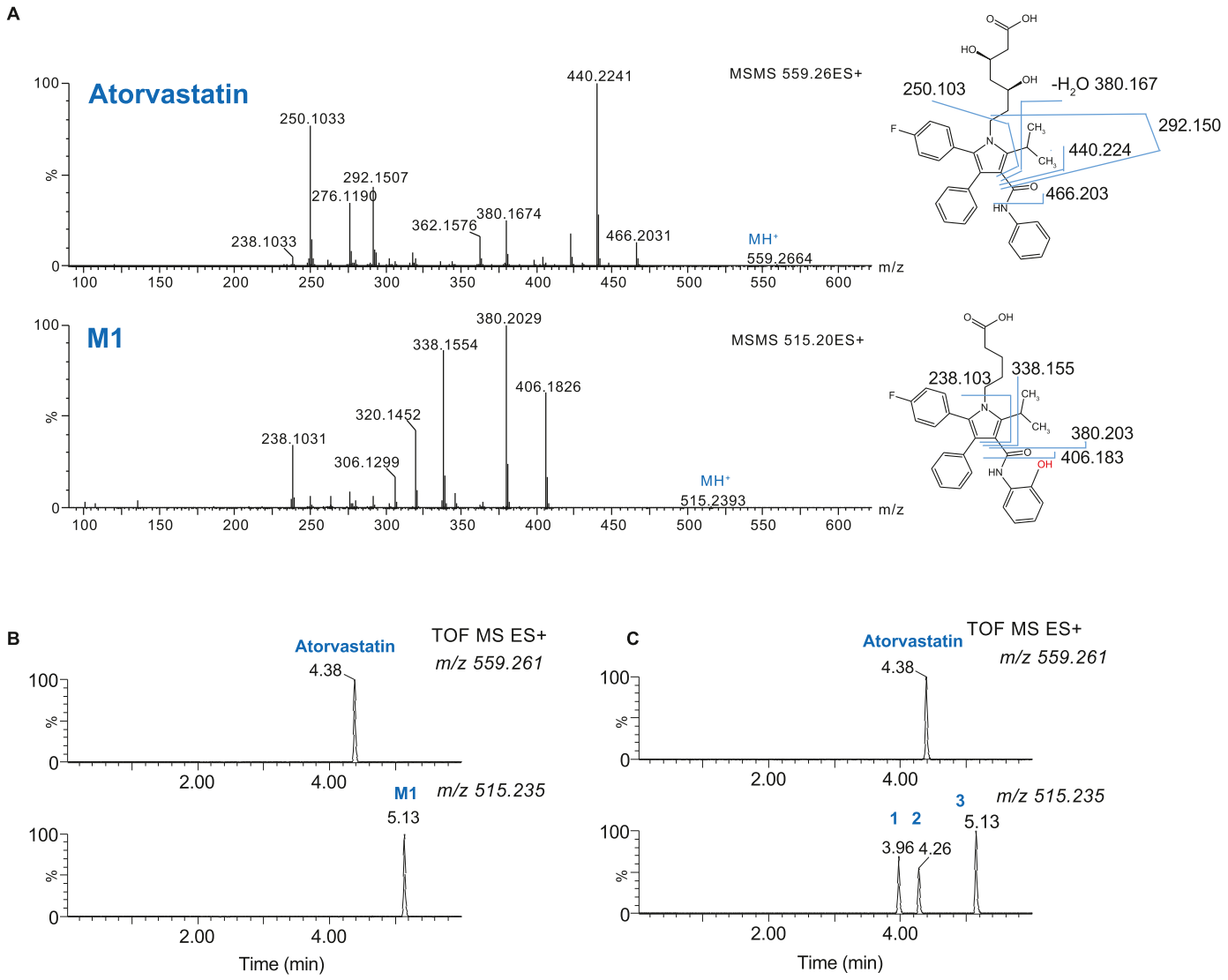

Fig. 1. A: Tandem mass spectrometry (MS/MS) product ion spectra acquired by collision-induced dissociation of the respective MH^+ ions of atorvastatin (top) and metabolite M1 (bottom) from mouse plasma. B: UPLC–HRMS chromatograms of atorvastatin and its hydroxy β-oxidized metabolite M1 in mouse plasma. C: UPLC–HRMS chromatograms of spiked mouse plasma containing the synthesized references of atorvastatin and regioisomers of hydroxy β-oxidized atorvastatin. Peak 1 = *p*-hydroxy β-oxidized atorvastatin; Peak 2 = *m*-hydroxy β-oxidized atorvastatin; Peak 3 = *o*-hydroxy β-oxidized atorvastatin.
TOF MS ES+, time-of-flight mass spectrometry positive electrospray ionization; UPLC–HRMS, ultra-high-performance liquid chromatography–high-resolution mass spectrometry.

on day 22 using LC–MS/MS as described in the 'General quantitative bioanalysis' section.

Plasma serum amyloid A (SAA) levels were determined in the chronic dose groups on days –1, 10 and 22, using the mouse SAA enzyme-linked immunosorbent assay (ELISA) kit (TriDelta) according to the manufacturer's instructions. Plasma monocyte chemoattractant protein-1 (MCP-1) levels were determined in the chronic dose groups on day 22, using the DuoSet ELISA kit (R&D Systems) and following the manufacturer's instructions. Small intestine and colon infiltration of T cells, B cells and macrophages was assessed in the chronic dose groups on day 22, immunohistochemically in 5 μm sections from formalin-fixed, paraffin-embedded Swiss rolls. FACS analysis of peripheral blood mononuclear cells (PBMCs) in the chronic dose groups on day 22 was performed using the following antibodies: CD11b-FITC, CD62L-PerCP-Cy5.5, CD19-APC-eFluor780, CD44-PE, Ly6C-APC, CD3-eFluor450 and CD11c-PE-Cy7 (all from Affymetrix eBioscience). Plasma levels of the cytokines were assessed using the V-PLEX pro-inflammatory panel 1 mouse kit (Meso Scale Discovery).

Pharmacokinetic analysis of mice data

Pharmacokinetic analysis was conducted as described in the 'Overall pharmacokinetic analysis'section, based on $AUC_{0–10h}$ or $AUC_{0–24h}$ for atorvastatin and metabolite M1, respectively. Non-compartmental pharmacokinetic analyses were performed group-wise using the median measured value at each time point. Values below the lower limit of quantification (LLOQ) were substituted with LLOQ/2.

Statistical analysis of mice data

The difference in exposure between the administrations with and without mineral oil was assessed by calculation of the ratios for the estimated median C_{max}, $AUC_{0–t}$ and AUC_{inf} for atorvastatin and metabolite M1. Statistical analyses of the pharmacodynamic data were performed using SPSS Statistics Version 25.0 (IBM Corporation). To compare the atorvastatin single dosing groups, a one-way analysis of variance test for multiple comparisons was used with Dunnett or Bonferroni correction. To compare the atorvastatin repeat-dosing groups, a Student's *t*-test for independent samples was used. A p value < 0.05 was

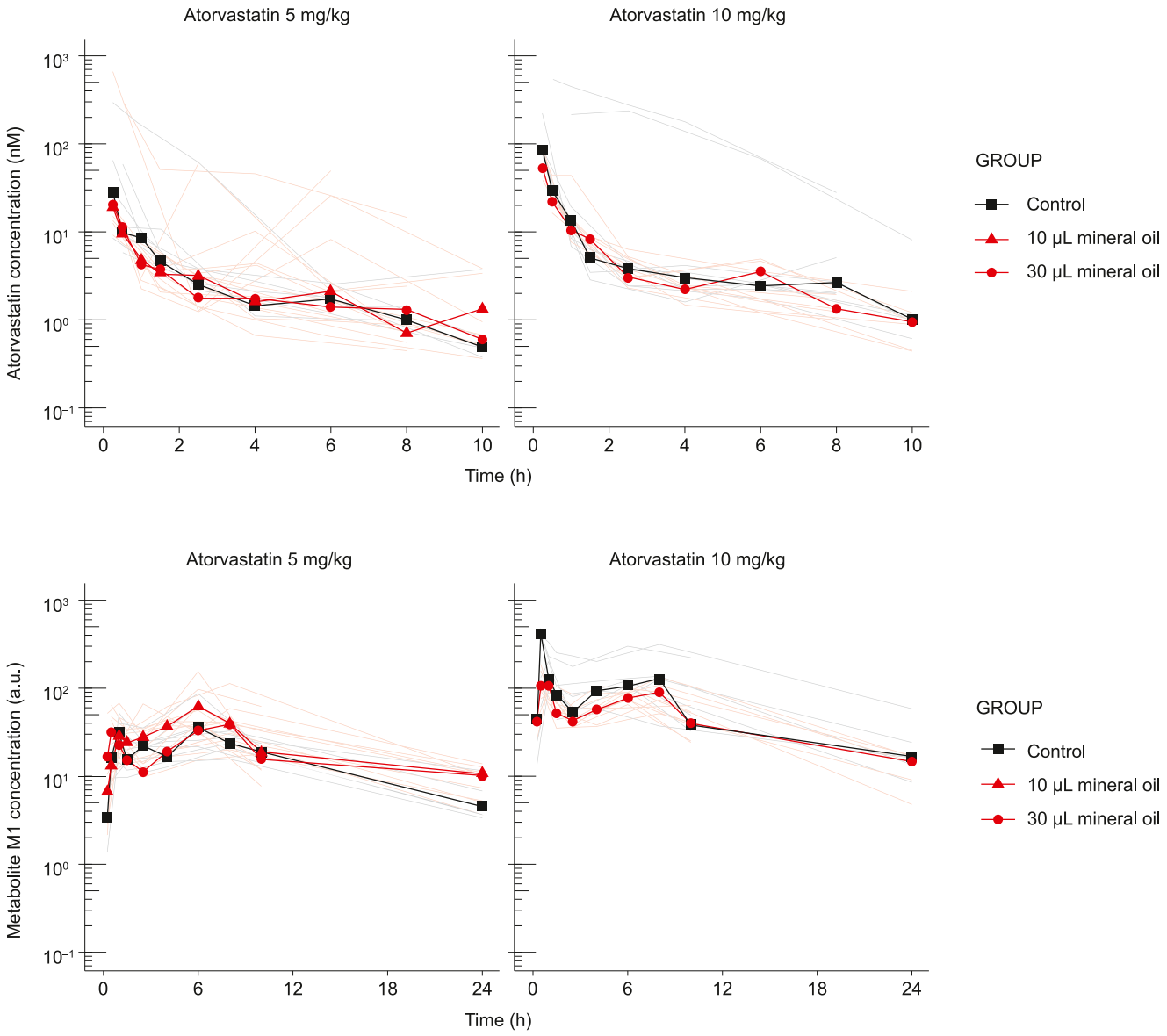

Fig. 2. Median plasma concentrations versus time profiles of atorvastatin (top) and metabolite M1 (bottom) following administration of a single dose of atorvastatin 5 mg/kg and 10 mg/kg without (black squares) or together with 10 µL (red triangles) or 30 µL (red circles) mineral oil in mice. Individual plasma concentrations of atorvastatin without (grey lines) and with (red lines) 10 µL or 30 µL mineral oil.

considered statistically significant.

Pharmacokinetics in dogs

Experimental design

The study had a single-group, two-period, cross-over-sequence design. In the first study period, dogs were administered atorvastatin (1 × 10 mg tablet) plus pravastatin (1 × 40 mg tablet) without mineral oil. Following a 1-week washout, dogs were administered atorvastatin (1 × 10 mg tablet) plus pravastatin (1 × 40 mg tablet) with 2 g mineral oil (2 × 1 g capsule). In the second study period, approximately 3 months later, each dog was administered atorvastatin (1 × 10 mg tablet) plus pravastatin (1 × 40 mg tablet) with 2 g mineral oil (2 × 1 g capsule). Following a 1-week washout, dogs were administered atorvastatin (1 × 10 mg tablet) plus pravastatin (1 × 40 mg tablet) without mineral oil. On the days before statins were co-administered with mineral oil, each dog received 2 × 1 g capsules of mineral oil in the morning and 2 × 1 g capsules of mineral oil in the afternoon. Each administration was followed by 10 mL of tap water given by a syringe to the dog's mouth. Animals were dosed with the statins in the mornings. The dogs were fasted 20 hours prior and 4 hours post dosing of the statins. When dogs received atorvastatin plus pravastatin tablets (with or without mineral oil capsules), this was followed by 10 mL of tap water given by a syringe to the dog's mouth and then an additional 50 mL of tap water given via an orogastric tube. Blood samples for pharmacokinetic analysis were collected from a peripheral vein in the front leg, back leg or neck before dosing, and at 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8 and 24 hours after dosing.

Sample analysis

Plasma concentrations of atorvastatin, its main metabolites in dog and pravastatin were assessed using LC–MS/MS as described in the 'General quantitative bioanalysis' section.

Pharmacokinetic analysis of dog data

Pharmacokinetic analysis was conducted as described in the 'Overall pharmacokinetic analysis' section, based on AUC from zero to time 8 hours ($AUC_{0–8h}$) for atorvastatin, *o*-hydroxy atorvastatin and

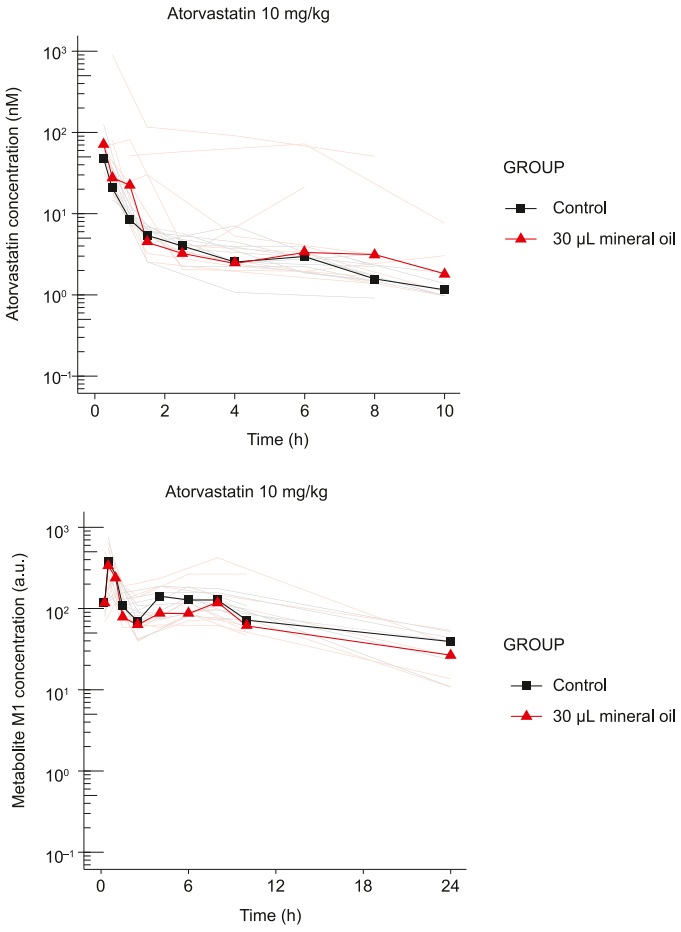

Fig. 3. Median plasma concentrations versus time profiles of atorvastatin (top) and metabolite M1 (bottom) following 22 days repeat administration of atorvastatin 10 mg/kg without (black squares) or together with (red triangles) 30 µL mineral oil in mice. Individual plasma concentrations of atorvastatin without (grey lines) and with (red lines) mineral oil.

pravastatin. For *p*-hydroxy atorvastatin, an AUC from zero to time 6 hours ($AUC_{0–6h}$) was assessed.

Statistical analyses of dog data

The difference between the administrations with and without mineral oil was assessed by calculation of 95% CI of the ratios for C_{max}, $AUC_{0–t}$ and AUC_{inf}. A 95% CI not containing the value '1.0' corresponded to a statistically significant difference at a $p < 0.05$ level.

Results

Metabolite characterization in mice

Characterization of metabolite M1 in plasma from mice was facilitated by using the three synthetic hydroxy β-oxidized atorvastatin regioisomers, all of which were fully characterized using ^{1}H and ^{19}F nuclear magnetic resonance spectroscopy and HRMS. Supplementary data Figure S1 summarizes the general synthetic strategy for the desired hydroxy β-oxidized atorvastatin regioisomers and nuclear magnetic resonance data for the individual regioisomers. The synthetic strategy applied made an unambiguous determination of the position of the hydroxy group on the aniline moiety straightforward. The precursor ions ($[MH]^+$) of atorvastatin, metabolite M1 and hydroxy β-oxidized atorvastatin were observed at *m/z* 559.2610, 515.2349 and *m/z* 515.2350, respectively. The proposed elemental composition of metabolite M1 was $C_{31}H_{31}FN_2O_4$ (mass error 1.6 ppm). HRMS/MS spectra of atorvastatin, metabolite M1 and regioisomers of hydroxy β-oxidized atorvastatin in positive MS mode were characterized with fragment ions derived from the neutral loss of the aniline as shown in Fig. 1A and Fig. 1B, respectively. There were no diagnostic fragments to distinguish the regioisomers of the hydroxylated aniline moiety, consistent with a previous report (Black et al., 1998). However, the availability of the synthetic reference standards allowed for their separation chromatographically (Fig. 1C). Atorvastatin eluted at retention time (RT) = 4.38 minutes, and *p-*, *m-* and *o*-hydroxy β-oxidized atorvastatin eluted at RT = 3.96, 4.26 and 5.13 minutes, respectively. By comparison, the analysis of plasma extracts from mice dosed with atorvastatin showed elution of metabolite M1 with RT matching that of *o*-hydroxy β-oxidized atorvastatin as shown in Fig. 1B and Fig. 1C. Collectively, the chemical structure of metabolite M1 was therefore confirmed chromatographically and mass spectrally to be *o*-hydroxy β-oxidized

Table 1
Non-compartmental pharmacokinetic parameters and ratios for atorvastatin and its metabolite M1 in mouse following administration of atorvastatin 5 mg/kg or 10 mg/kg with or without mineral oil.

Measured compound	**Atorvastatin**							**Metabolite M1**[a]						
Study	Single dose					Repeated dose		Single dose					Repeated dose	
Atorvastatin	5 mg/kg			10 mg/kg		10 mg/kg		5 mg/kg			10 mg/kg		10 mg/kg	
Mineral oil, µL	0	10	30	0	30	0	30	0	10	30	0	30	0	30
C_{max}, nM or a.u.[a]	28.5	19.2	20.6	85.8	53.5	49.0	77.0	35.8	62.5	38.6	415	108	580	340
t_{max}, h	0.25	0.25	0.25	0.25	0.25	0.25	0.25	6.0	6.0	8.0	0.50	1.0	0.50	0.50
$AUC_{0–t}$, h × nM or h × a.u.[a]	29.8	26.0	25.3	62.6	50.4	47.2	64.7	365	555	416	1360	1010	2050	1570
AUC_{inf}, h × nM or h × a.u.[a]	33.0	28.5	28.7	71.4	57.9	53.6	80.8	410	708	566	1500	1150	2650	1930
$t_{1/2}$, h	3.7	3.1	3.6	4.5	4.6	4.0	6.7	6.8	10	11	6.7	7.1	11	9.4
C_{max} ratio[b]		0.67	0.72		0.62		1.6		1.7	1.1		0.26		0.59
$AUC_{0–t}$ ratio[b]		0.87	0.85		0.81		1.4		1.5	1.1		0.74		0.77
AUC_{inf} ratio[b]		0.86	0.87		0.81		1.5		1.7	1.4		0.77		0.73

AUC, area under the plasma concentration–time curve; $AUC_{0–t}$, AUC from zero to time t (t = 10 h for atorvastatin and 24 h for the metabolite M1); AUC_{inf}, AUC from zero to infinity; C_{max}, maximum (peak) plasma concentration; $t_{1/2}$, terminal half-life; t_{max}, time to reach maximum plasma concentration.

[a] Units for C_{max} and AUC for M1 are arbitrary because atorvastatin was used for calibration.

[b] The C_{max} and AUC ratios were calculated as follows: with mineral oil/without mineral oil.

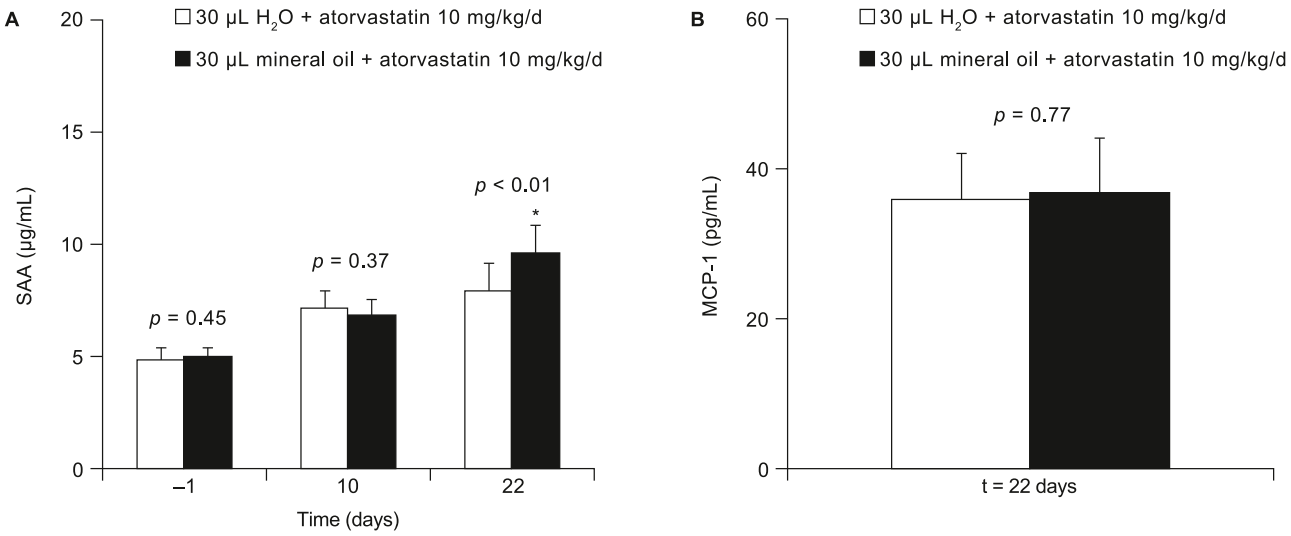

Fig. 4. Mean plasma levels of A: SAA on days –1, 10 and 22, and B: MCP-1 on day 22, after 21 days of daily dosing of atorvastatin 10 mg/kg without or with co-administration of 30 µL mineral oil in mice.
MCP-1, monocyte chemoattractant protein-1; SAA, serum amyloid A.

atorvastatin.

Pharmacokinetics and pharmacodynamics in mice

Animals

All mice appeared normal, behaved normally and showed no signs of discomfort. Body weights remained similar between groups during the run-in period in mice receiving single dose atorvastatin and during the 22-day dosing period in mice receiving repeat once-daily dosing.

Atorvastatin and main metabolite pharmacokinetics in mice

Bioanalysis of atorvastatin and metabolite M1 was performed in mice according to the methods described in Supplementary data Table S1. The LLOQ of the assay ranged between 0.214 nM and 1.07 nM, and precision and accuracy of the calibration standards were within 8.3% CV and 18% of nominal concentration, respectively. Concentrations for M1 are reported as arbitrary units because atorvastatin was used for calibration. Still, concentrations of M1 were minimally affected when measured against either the calibration curve of atorvastatin or that of M1 (error within 9.5%), indicating that the quantification of M1 using atorvastatin as reference was reliable.

Figs. 2 and 3 show median plasma concentrations of atorvastatin and metabolite M1 after a single dose of atorvastatin at 5 mg/kg or 10 mg/kg with or without co-administration of 10 µL or 30 µL mineral oil (Fig. 2), and after 21 days of daily dosing of atorvastatin at 10 mg/kg with or without co-administration of 30 µL mineral oil (Fig. 3), respectively, in mice. Due to the limited number of sampling time points from each mouse (5 time points), a full exposure profile could only be constructed with samples from several animals. Therefore, a single non-compartmental analysis was performed for each group, resulting in a point estimate without statistical uncertainty. Median values at each time point were chosen as the most representative for assessing the effects of mineral oil in this data set, because the presence of a few, most likely random outliers skewed the mean and log mean values.

Pharmacokinetic parameters of atorvastatin in plasma after single dose and repeat dosing are shown in Table 1. The single-dose experiments showed a trend to reduce atorvastatin exposure in the mineral oil groups (ratio of AUC_{0-inf} 0.81–0.87). However, the repeat dose experiment resulted in increased exposure in the mineral oil group (ratio of AUC_{0-inf} 1.5). The exposure of the metabolite M1 was consistently increased for the 5 mg/kg atorvastatin dose groups and reduced for the 10 mg/kg atorvastatin dose groups when co-administered with mineral oil, with AUC_{0-inf} ratios ranging from 0.73 to 1.7 (Table 1). Similarly, the ratio for C_{max} with and without mineral oil administration varied between dose groups and regimens, with ratios ranging from 0.62 to 1.6 for atorvastatin and 0.26 to 1.7 for M1. Based on these data we conclude that there are no consistent differences in plasma exposure of atorvastatin when administered alone or co-administered with mineral oil in mice.

Inflammatory markers

Plasma SAA levels on day 22 were statistically significantly higher after daily dosing of atorvastatin 10 mg/kg with co-administration of 30 µL mineral oil than without mineral oil co-administration (mean [standard deviation (SD)]: 9.6 [1.3] µg/mL vs 7.9 [1.2] µg/mL, $p < 0.01$) (Fig. 4). Plasma SAA levels on days –1 and 10 with mineral oil versus without mineral oil were not significantly different. Plasma MCP-1 levels on day 22 did not differ significantly with mineral oil versus without mineral oil co-administration.

The subset of $C62L^{high}$ B cells on day 22 was statistically significantly higher after daily dosing of atorvastatin 10 mg/kg with co-administration of 30 µL mineral oil than without mineral oil co-administration (mean [SD] percentage of B cells: 18 [7]% vs 12 [3]%, $p = 0.04$) (Fig. 5). Conversely, the subset of $C62L^{low}$ B cells on day 22 was statistically significantly lower after 21 days of daily dosing of atorvastatin 10 mg/kg with co-administration of 30 µL mineral oil than without mineral oil co-administration (mean [SD] percentage of B cells: 81 [7]% vs 87 [4]%, $p < 0.05$). No statistically significant differences were observed for PBMC composition overall, and by T-cell and monocyte subsets.

Fig. 6 shows plasma levels of interleukin-5 (IL-5), IL-6, IL-10, keratinocyte chemoattractant/growth-regulated oncogene chemokine (KC/GRO) and tumour necrosis factor-α (TNF-α). There were no substantial differences on days 10 and 22 in repeat daily dosing groups that received atorvastatin with or without mineral oil. Cytokine levels were near the LLOQ for IL-5, IL-6 and IL-10, and were below the LLOQ for interferon-γ (IFN-γ), IL-1β, IL-2, IL-4 and IL-12.

There were no statistically significant differences in the mean number of T cells, B cells or macrophages per mm^2 of small intestine or colon after repeat dosing of atorvastatin 10 mg/kg with or without co-administration of 30 µL mineral oil (Fig. 7).

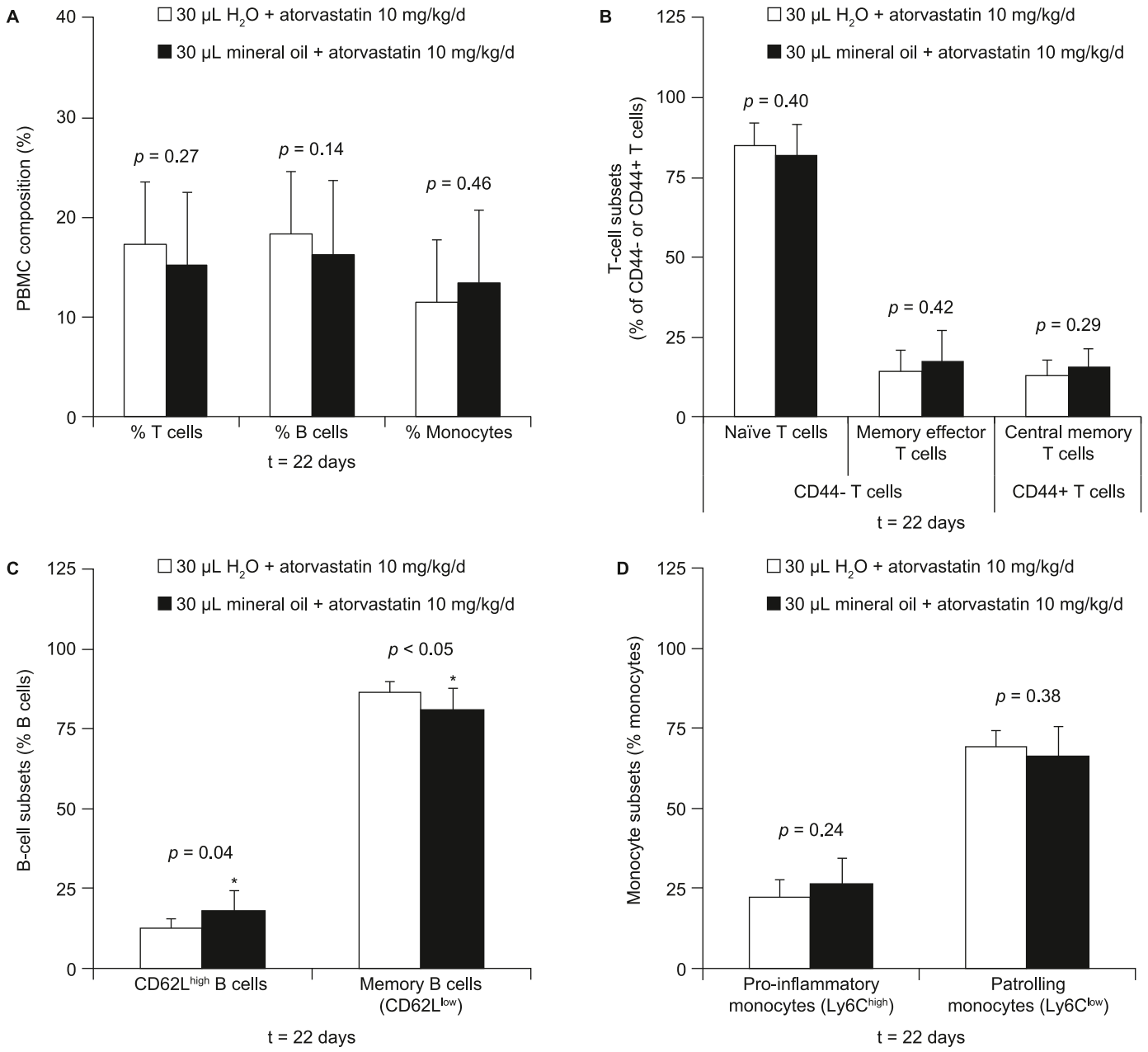

Fig. 5. Mean plasma PBMC composition A: overall, and of B: T-cell subsets, C: B-cell subsets and D: monocyte subsets on day 22, after 21 days of daily dosing of atorvastatin 10 mg/kg without or with co-administration of 30 µL mineral oil in mice.
PBMC, peripheral blood mononuclear cell.

Pharmacokinetics in dogs

Animals

Animals appeared healthy, except for a mild nose drip and wet nose that occurred a short time after dosing; these were observed for a limited duration of time during continuous monitoring by the animal staff and had no impact on the health of the animals. Nasopharyngitis is a common side effect for atorvastatin in humans (Pfizer, 2019).

Atorvastatin, o- and p-hydroxy atorvastatin, and pravastatin pharmacokinetics in dogs

Bioanalysis of plasma concentrations of atorvastatin and *o*- and *p*-hydroxy atorvastatin and pravastatin in dogs was achieved as described in Supplementary data Table S1. The quality control results for atorvastatin and *o*- and *p*-hydroxy atorvastatin were within ±15% or better of expected values, confirming that the assay performed within acceptable criteria. For pravastatin, the samples were analysed separately, and for both study periods LLOQ was 2.03 nM, precision for calibration standards was within 8.6% CV and accuracy was within ±11% of nominal concentration.

Fig. 8 shows geometric mean plasma concentrations versus time profiles of atorvastatin, *o*-hydroxy atorvastatin, *p*-hydroxy atorvastatin and pravastatin following co-administration of atorvastatin 10 mg and pravastatin 40 mg tablets with or without mineral oil capsules in dogs. Supplementary data Figure S2 depicts the plasma concentrations versus time profiles separately for each individual animal. Pharmacokinetic parameters are listed in Table 2 and Supplementary data Table S2. No statistically significant differences were observed in C_{max}, AUC_{0-8h} or AUC_{inf} of atorvastatin or pravastatin when given together with mineral oil capsules compared with when given without mineral oil capsules

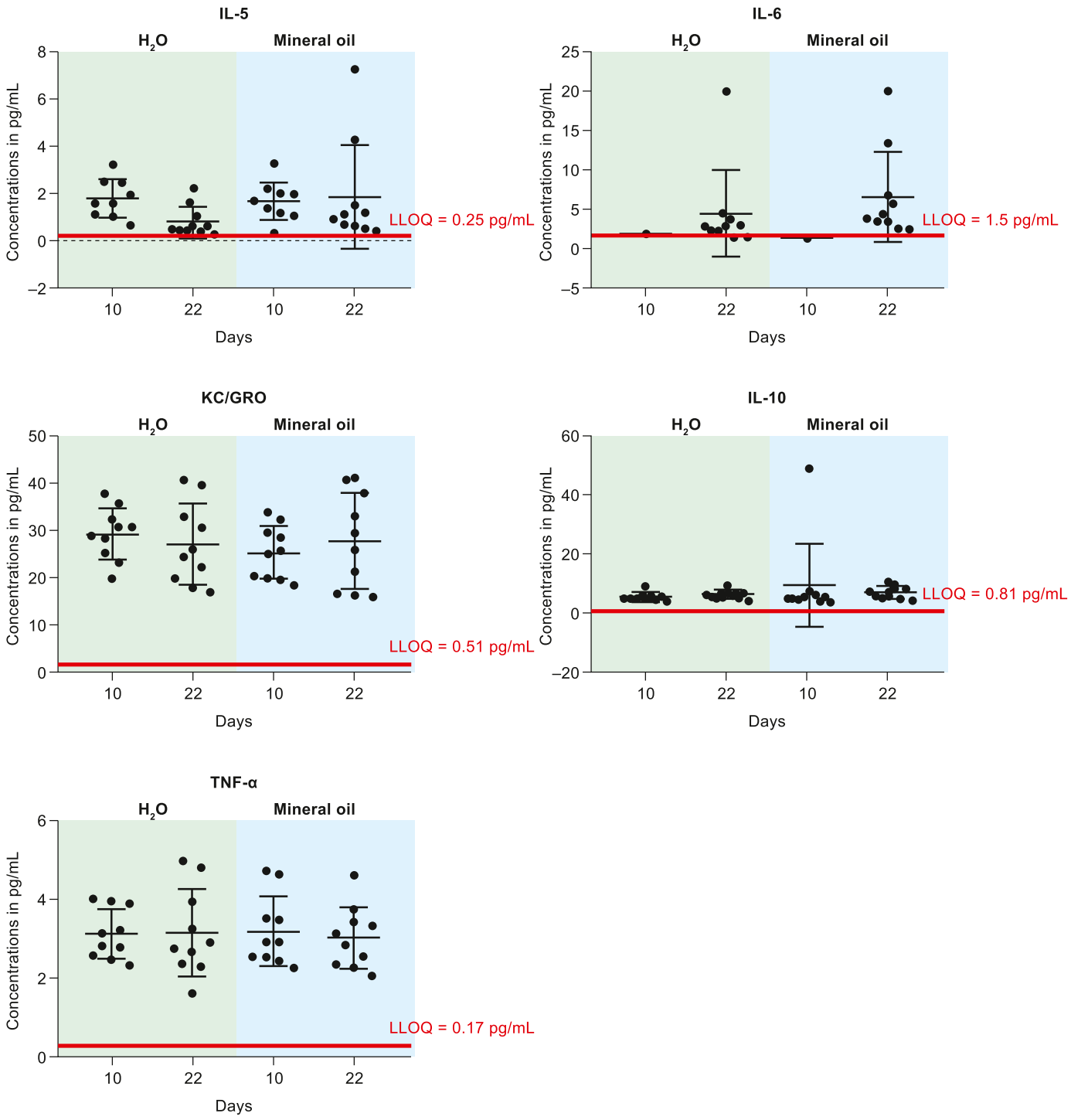

Fig. 6. Plasma levels of IL-5, IL-6, KC/GRO, IL-10 and TNF-α on days 10 and 22 in mice chronically dosed with atorvastatin 10 mg/kg daily without or with co-administration of 30 μL mineral oil for 21 days.
H_2O, purified water; IL, interleukin; KC/GRO, keratinocyte chemoattractant/growth-regulated oncogene chemokine; LLOQ, lower limit of quantification; TNF-α, tumour necrosis factor-α.

(Table 2). In addition, no statistically significant differences were observed in C_{max} or AUC_{0-t} for the two atorvastatin metabolites, *o*-hydroxy atorvastatin and *p*-hydroxy atorvastatin, when given together with mineral oil capsules compared with when given without mineral oil capsules (Supplementary data Table S2).

Discussion

Little information is available on the biological effects of orally administered mineral oil and whether it affects the pharmacokinetics of statins. We thus conducted this work in mice and dogs to assess if mineral oil has biological activity, either by affecting statin absorption in the gastrointestinal tract or by a direct, pro-inflammatory mechanism. Under our experimental conditions, following single dosing or 3-week

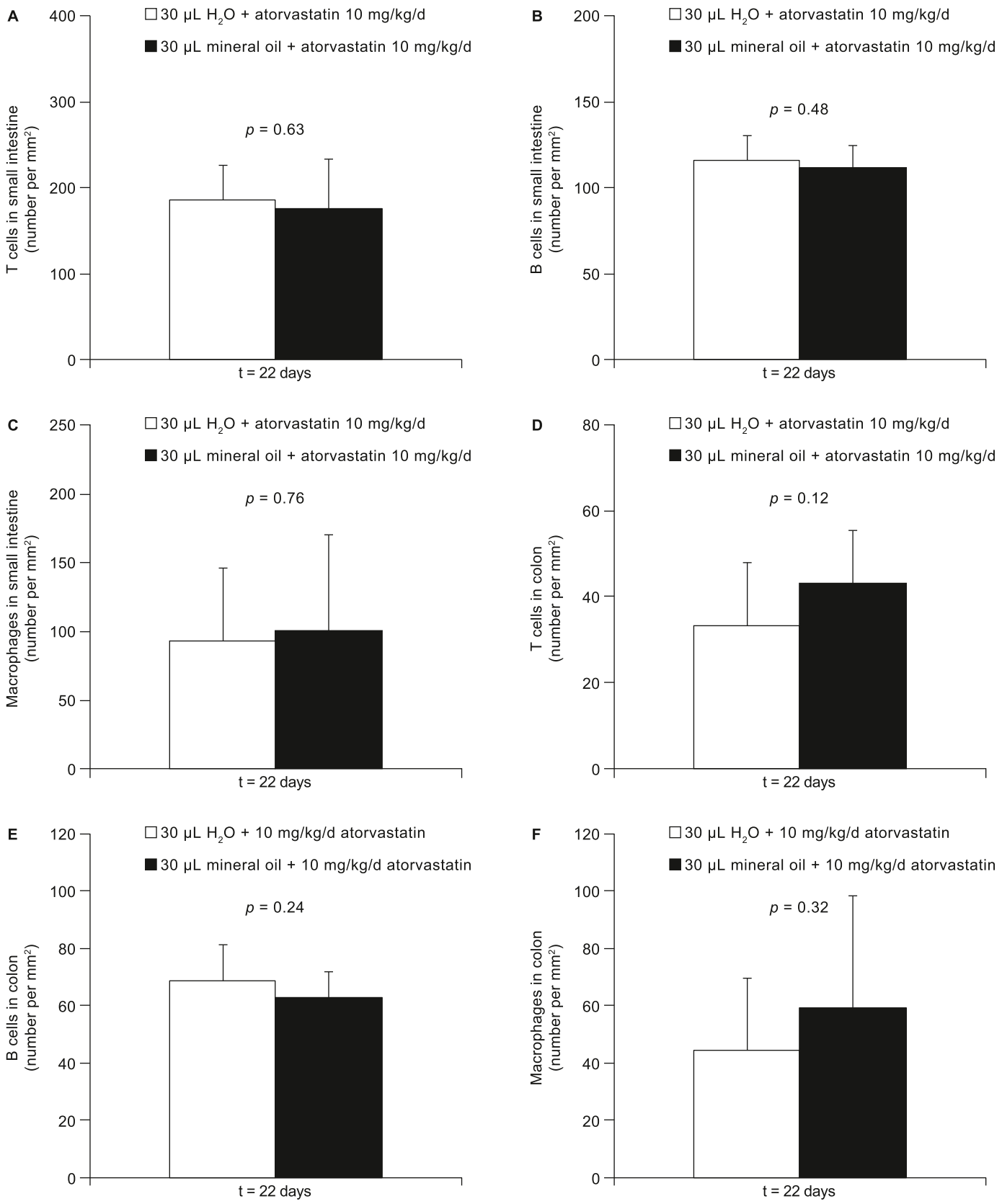

Fig. 7. Mean number of T cells, B cells and macrophages per mm^2 in small intestine and colon after repeat dosing with daily atorvastatin with or without mineral oil in mice.

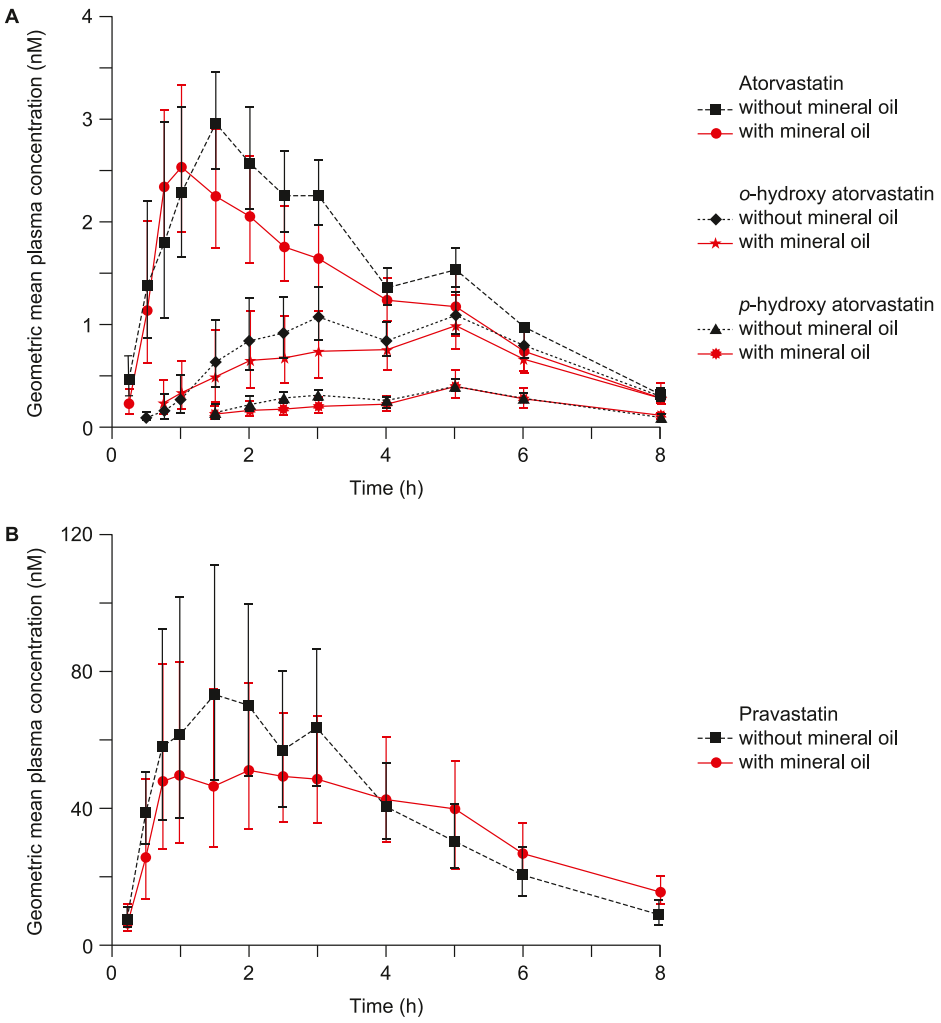

Fig. 8. Geometric mean (± standard error) plasma concentrations versus time profiles of atorvastatin, *o*-hydroxy atorvastatin and *p*-hydroxy atorvastatin (A) and pravastatin (B) in Labrador dogs following administration of atorvastatin 10 mg and pravastatin 40 mg tablets with (red symbols) or without (black symbols) mineral oil capsules.

repeat daily dosing of atorvastatin, we observed no consistent differences in plasma exposure (C_{max} or AUC) of atorvastatin or its main metabolite M1 in mice when the statin was administered with or without mineral oil. Similarly, in dogs there was no evidence that C_{max} or AUC of atorvastatin, its main hydroxy metabolites or pravastatin were significantly affected by the co-administration of mineral oil. Therefore, the results did not validate our first hypothesis, which proposed that mineral oil affects the absorption of statins (Prescriber's Digital Reference, 2021; Steigmann et al., 1952).

On the other hand, the results of the present work support our second hypothesis, that chronic use of mineral oil causes direct, low-grade inflammation. Increased inflammatory signals were observed after 3 weeks of mineral oil co-administration in mice. Plasma SAA levels were statistically significantly increased in mice co-administered mineral oil compared with mice not co-administered mineral oil. SAA is an acute phase marker that responds rapidly, similarly to C-reactive protein, and is secreted mainly by the liver during the acute phase of inflammation. There was a statistically significant increase in circulating L-selectin-expressing ($C62L^{high}$) B cells and a corresponding, statistically significant decrease in circulating memory ($C62L^{low}$) B cells in mice co-administered mineral oil compared with mice not co-administered mineral oil. CD62L is shed from lymphocytes upon activation, and the decrease in circulating memory (activated) B cells may thus indicate increased tissue infiltration with mineral oil, although no effects on B cell numbers in small intestine and colon were observed.

The doses of atorvastatin, pravastatin and mineral oil used in our studies translate well to the clinical setting in humans. The mice received atorvastatin at a dose of either 5 or 10 mg/kg/day. These doses translate to approximately 32.5 and 65 mg of atorvastatin per day, respectively, for an 80 kg human, using a simplified calculation based on body surface area as accepted by the FDA as a guide (Nair and Jacob, 2016). Thus, the doses used are similar to the highly efficacious 40 and 80 mg/day doses of atorvastatin used in the clinic (Jones et al., 1998), which result in similar decreases in plasma cholesterol levels, of about 40–50%, in mice and humans (Jones et al., 1998; van De Poll et al., 2001; Pouwer et al., 2020). The 30 μL/day dose of mineral oil given to mice translates to 4 mL/day in humans, based on the difference in intestinal surface area between mice and humans (human dose × intestinal surface area in mouse divided by intestinal surface area in humans) (Casteleyn et al., 2010). Alternatively, based on the simplified body surface area calculation (Nair and Jacob, 2016), the mineral oil dose of 4 mL/day used as a placebo in clinical trials equates to 12 μL/day for a 20 g mouse. We used 10 μL as a 0.5log lower dose as compared with the 30 μL dose. For the dog study, the doses of atorvastatin (10 mg) and pravastatin (40 mg) correspond to a moderate intensity statin therapy in humans (Stone et al., 2014). Both statin and mineral oil doses were in line with those used in a recent omega-3 fatty acid trial (Bhatt et al., 2019).

If the low-grade inflammation seen with oral mineral oil administration in our experiments in mice translates into the human setting,

Table 2
Non-compartmental pharmacokinetic parameters and ratios of atorvastatin and pravastatin in Labrador dogs following co-administration of atorvastatin 10 mg and pravastatin 40 mg tablets with or without mineral oil capsules.

	Atorvastatin		Pravastatin	
	Without mineral oil	With mineral oil	Without mineral oil	With mineral oil
Geometric mean C_{max}, nM	3.74	3.00	98.7	90.4
(range)	(2.17–10.0)	(1.51–7.77)	(29.4–547)	(25.3–362)
Median t_{max}, h	1.50	1.75	1.75	1.50
(range)	(0.5–5.0)	(0.5–5.0)	(0.75–3.0)	(0.75–5.0)
Geometric mean AUC_{0-8h}, nM × h	13.0	10.6	324	332
(range)	(8.41–20.5)	(5.38–25.9)	(162–1420)	(98.1–871)
Geometric mean AUC_{inf}, nM × h	14.0	12.3	366	429
(range)	(8.99–20.7)	(5.69–29.6)	(208–1650)	(108–1050)
Geometric mean $t_{1/2}$, h	1.4	2.3	2.1	3.7
(range)	(0.9–3.4)	(1.0–8.0)	(1.0–3.6)	(2.0–11)
Geometric mean C_{max} ratio[a]	0.80		0.92	
(95% CI)	(0.45–1.4)		(0.61–1.4)	
Geometric mean AUC_{0-8h} ratio[a]	0.82		1.0	
(95% CI)	(0.49–1.4)		(0.70–1.5)	
Geometric mean AUC_{inf} ratio[a]	0.88		1.2	
(95% CI)	(0.49–1.6)		(0.76–1.8)	

AUC, area under the plasma concentration–time curve; AUC_{0-8h}, AUC from zero to time 8 h; AUC_{inf}, AUC from zero to infinity; CI, confidence interval; C_{max}, maximum (peak) plasma concentration; $t_{1/2}$, terminal half-life; t_{max}, time to reach maximum plasma concentration.

[a] The overall C_{max} and AUC ratios (with mineral oil/without mineral oil) were calculated by first calculating a ratio for each dog and then averaging across dogs.

then this could have led to low-grade inflammation and thus to an increased cardiovascular risk when used as a placebo in clinical trials (Ridker et al., 1997; Ridker et al., 2000; Ference et al., 2017). The results from the present work emphasize the importance of the appropriate selection of oil in the placebo arm of trials of omega-3 fatty acids and other oil-based drug formulations, and support the need for more data to confirm the effects of omega-3 fatty acids on cardiovascular outcomes. Several trials that are ongoing, around completion or closing using either mineral oil or corn oil as a placebo may elucidate further the effects of omega-3 fatty acids on cardiovascular risk (Laake et al., 2014; Budoff et al., 2018; Nicholls et al., 2018).

Our work has several key strengths. We assessed the effects of mineral oil on statin pharmacokinetics and inflammation after single and repeated daily dosing in mice, and confirmed our pharmacokinetic results in a different, larger animal by studying the effects after single dosing in dogs. As part of the work in mice, the atorvastatin main metabolite, M1, was fully characterized. Metabolite M1 had been previously identified but only partially characterized (Black et al., 1998). The structure of metabolite M1 was confirmed to be *o*-hydroxy β-oxidized atorvastatin by comparison of the metabolite M1 to a corresponding synthetic reference standard. Availability of the three regioisomers of hydroxy β-oxidized atorvastatin as reference standards combined with chromatographic and mass spectral analyses were key for this unequivocal characterization of metabolite M1. The result allowed further assessment and evaluation of the absorption of atorvastatin in mouse, by taking into account effects on both the exposure of atorvastatin and that of the major metabolite M1. The combined pharmacokinetic results for atorvastatin and M1 suggest that there were no consistent differences between groups when co-administering atorvastatin with mineral oil, compared with administering atorvastatin alone, with AUC and C_{max} ratios both below and above 1. Any observed differences in individual groups were, therefore, likely not the result of mineral oil effects on drug absorption, but rather due to the small sample size and the intra- and inter-individual variations. Different from mice, the major circulating metabolites in dogs were *p*- and *o*-hydroxy atorvastatin. The simultaneous quantification of atorvastatin and these two active metabolites, using a methodology based on those that have been described previously (Jemal et al., 1999; Yacoub et al., 2013), strengthened the pharmacokinetic analysis conducted in dogs. Assessment of pravastatin metabolites in dog plasma was not necessary because detection levels of pravastatin were sufficiently high and well above its LLOQ. A limitation of our work is that oral administration by gavage of an equivalent human dose volume of neat mineral oil was not possible in mice because of the very low volume required and, therefore, a mineral oil-in-water emulsion was administered instead at the low (10 μL) dose and pure mineral oil at the higher (30 μL) dose.

Conclusion

Our work showed that mineral oil did not affect the absorption of atorvastatin, a lipophilic statin, or pravastatin, a hydrophilic statin, in mice or dogs under the study conditions used. However, the results showed that chronic oral administration of mineral oil can cause low-grade inflammation in mice. The findings add to the concern over whether mineral oil is an appropriate placebo for use in clinical studies of omega-3 fatty acids or oil-based formulations of other drugs. This warrants further investigation.

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Author contributions

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Elsbet J. Pieterman: conceptualization; formal analysis; project administration; supervision; writing – review & editing.

Hans M. G. Princen: conceptualization; supervision; writing – review & editing.

Linnéa Bergenholm: conceptualization; methodology; formal analysis; visualization; writing – review & editing.

Eva Lundborg: investigation; methodology; formal analysis; writing – review & editing.

Anders Cavallin: methodology; formal analysis; writing – review & editing.

Magnus J. Johansson: conceptualization; investigation; methodology; writing – review & editing.

Glen Hawthorne: investigation; writing – review & editing.

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Maria Hammarberg: methodology; investigation; writing – review & editing.

XueQing Li: methodology; formal analysis; writing – review & editing.

Annica Jarke: methodology; writing – review & editing.

Jonathan Bright: methodology; formal analysis; writing – review & editing.

Lena Svensson: methodology; formal analysis; writing – review & editing.

Rasmus Jansson-Löfmark: conceptualization; writing – review & editing.

Bertil Abrahamsson: conceptualization; writing – review & editing.

Rahul Agrawal: writing – review & editing; conceptualization; supervision.

Eva Hurt-Camejo: conceptualization; funding acquisition; investigation; supervision; writing – review & editing.

Competing interests

VSG, AC, MJJ, GH, LB, AB, EL, MH, XQL, AJ, JB, LS, RJL, BA, RA and EHC are employees of AstraZeneca and hold shares in AstraZeneca. EJP and HMGP are employees of contract facilities at TNO-Metabolic Health Research, which received funding from AstraZeneca for contract services.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ejps.2021.105776.

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