Antiretroviral therapy with or without protease inhibitors impairs postprandial TAG hydrolysis in HIV-infected men

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Mechanisms underlying the lipodystrophy syndrome associated with antiretroviral therapy (ART) for HIV infection are not completely understood. We investigated the effect of ART on blood lipid concentrations in the fasting state and after consumption of a meal containing [1-13C]palmitic acid in HIV-positive men receiving nucleoside reverse transcriptase inhibitors (NRTI, n 7), NRTI combined with protease inhibitors (PI; NRTIPI, n 6), in HIV-positive but therapy-naïve men (noART, n 5) and in HIV-seronegative men (controls, n 6). HIV-positive subjects had higher fasting TAG concentrations and resting energy expenditure than controls. Subjects receiving NRTIPI therapy had higher fasting NEFA concentrations than the other groups. There were no significant differences in postprandial lipid metabolism between noART subjects and controls. NRTI therapy impaired hydrolysis of meal-derived TAG, most evidently when combined with PI (the NRTIPI group). Accumulation of 13C-label in the NEFA fraction was not different between groups. In the NRTIPI group, fasting and postprandial NEFA concentrations were significantly higher than other groups. Postprandial glucose and insulin responses in HIV-positive subjects did not differ from controls. These findings suggest that ART dyslipidaemia is associated with impaired postprandial TAG clearance, which is exacerbated by NRTIPI therapy. If dyslipidaemia is to be minimised in ART, the specific adverse effects of particular combinations during the fed state should be considered.

HIV: Protease inhibitors: Nucleoside reverse transcriptase inhibitors: Dyslipidaemia: Stable isotopes

The mortality and morbidity due to HIV infection and resultant immunosuppression have been significantly reduced by the availability of potent combinations of antiretroviral therapy (ART)1–3. However, ART can induce metabolic and morphological changes due to perturbations of lipid metabolism4–5. Specifically, ART is associated with raised fasting TAG and HDL-cholesterol, VLDL TAG and intermediate-density lipoprotein concentration and lower LDL-cholesterol concentration6 leading to increased risk of CVD7,8. These changes in lipid metabolism are accompanied by redistribution of fat mass between adipose tissue depots, which because of perceived aesthetic changes may reduce treatment compliance9,10.

The effect of ART in seropositive subjects on the development of hypertriglyceridaemia is well recognised. The effect is variable and has been seen at its most severe extreme in earlier protease inhibitors (PI) therapy. The relative contribution of nucleoside reverse transcriptase inhibitors (NRTI) component within combination therapy is less well understood. Although the magnitude of the effect attributable to NRTI on its own may be smaller, there is increasing concern that when combined with PI, the interaction may result in greater perturbations in lipid metabolism than that seen with PI alone11. The mechanism underlying ART-associated dyslipidaemia is not well understood, but is reported to involve insulin resistance12 and increased activity of pro-inflammatory cytokines13, and in the fasted state, accelerated lipolysis11,14 and increased VLDL secretion14. In the fed state, marked perturbations in dietary TAG disposal, seen most obviously as enhanced postprandial lipaemia, are also believed to play an important role in the development of this condition14,15. Plasma TAG homeostasis in the fed state reflects the balance between the rates of lipid entering the circulation carried by chylomicron and VLDL, and capacity for clearance of endogenous and meal-derived TAG by lipoprotein lipase activity17 and the subsequent entrapment of fatty acids released by lipoprotein lipase activity by peripheral tissues18,19. Incomplete delipidation of lipoproteins and/or entrapment in the postprandial state would increase flux of fatty acids either as TAG in chylomicron remnants or as NEFA to the liver, which in turn drive VLDL TAG secretion. While there is some evidence of impaired clearance of TAG-rich lipoproteins20,21, the proposal that peripheral fatty acid trapping is impaired is supported only by indirect

Abbreviations: ART, antiretroviral therapy; AUC, area under the curve; NRTI, nucleoside reverse transcriptase inhibitors; NRTIPI, NRTI combined with protease inhibitors; REE, resting energy expenditure.

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Antiretroviral therapy and dyslipidaemia

Study subjects
Ethical approval was obtained from the Chelsea & Westminster Hospital Ethics Committee for all patients and from the Southampton and South West Hampshire Research Ethics Committee for the control subjects. Informed written consent was obtained from all participants. Nineteen asymptomatic HIV-positive men aged 20–54 years were recruited at the Chelsea & Westminster Hospital, London together with six age-matched HIV-negative male volunteers (controls) from the Institute of Human Nutrition, University of Southampton. The HIV-1-infected subjects were: n 6 ART-naive individuals (noART); n 6 individuals receiving PI-based ART (NRTIPi); n 7 PI-naive individuals receiving dual-NRTI regimens (n 7). Subject characteristics are summarised in Table 1 together with the individual antiretroviral regimens, viral load, CD4 and CD8 count. All patients on ART had self-reported moderate to severe peripheral fat loss and HIV lipodystrophy syndrome was confirmed by clinical examination(10). After completing the metabolic studies, one of the noART patients was found to have a malignancy and was withdrawn from the analysis. Patients in NRTIPi group had been on PI-based regimens for at least 12 months.

Postprandial study
The subjects avoided foods naturally enriched with 13C (e.g. maize products, cane sugar) for 2 d before the study and refrained from eating or drinking from 22.00 hours on the day before the study. Participants attended at 07.00 h and underwent anthropometric assessments and bioelectrical impedance (Bodystat 500 BIA, Bodystat Limited, Isle of Man, UK). REE and substrate oxidation were determined by indirect calorimetry (Bodystat 500 BIA, Bodystat Limited, Isle of Man, UK), and fatty acid methyl esters were prepared by incubation with 2 % (v/v) H2SO4 in methanol(26). Fasting venous bloods were drawn from an indwelling forearm cannula. Subjects consumed a standard test breakfast providing 3720 kJ and containing, 45 g lipid, 93 g carbohydrate and 33 g protein. This consisted of cereal, milk, bread, margarine and cheese together with a lipid:glucose:casein emulsion containing 700 mg [1-13C]palmitic acid ninety-nine atom per cent excess (Masstrace, Woburn, MA, USA)(23). Venous blood samples were collected at half-hourly intervals for 3 h and then hourly for the next 4 h. Measurement of gaseous exchange and expired air sampling was performed hourly for 7 h. The patients were only allowed to drink water and were required to remain seated or supine throughout the study period.

Materials and methods

Measurement of energy expenditure
REE and estimates of net lipid and carbohydrate oxidation were made from gaseous exchange measurements(24). Net lipid and carbohydrate oxidation over the 7 h postprandial period were determined as the area under the curve (AUC) from the time by oxidation plots, and expressed as the relative contribution made by carbohydrate and lipid oxidation towards energy expenditure.

Measurement of fatty acid β-oxidation
Enrichment of 13C in breath as 13CO2 was determined by continuous-flow-isotope ratio mass spectrometer (20–20 stable isotope analyser, Europa Scientific Ltd, Crewe, UK). The proportion of 13C-label excreted on breath as 13CO2 was expressed as a percentage of administered 13C-label per hour and as the cumulative percentage dose excreted over 7 h(23). From the whole-body net lipid oxidation and recovery of expired label, it was possible to determine the apparent oxidation of exogenous and endogenous lipid oxidation over the study period. Thus, endogenous lipid oxidation = net lipid oxidation from indirect calorimetry minus exogenous lipid oxidation from 13CO2 recovery on breath.

Measurement of total plasma lipids and of 13C-labelled fatty acid concentrations in plasma
Total TAG and NEFA concentrations were measured by summation of the concentrations of individual fatty acids in these pools using GC-combustion-isotope ratio MS (see below). This approach avoided the need to dilute plasma samples with high TAG concentrations, which would have been required if conventional colorimetric analysis had been used. Triheptadecanoin (100 μg) and heneicosanoic acid (30 μg) were added to plasma (0·8 ml) as internal standards to correct for recovery and tricosanoic acid methyl ester (1·13 atom %) added to measure isotopic enrichment and total lipids were extracted from plasma with chloroform and methanol(25). TAG and NEFA were purified by solid-phase extraction using 100 mg aminopropylsilica cartridges (Varian, Walton-on-Thames, Surrey, UK) and fatty acid methyl esters were prepared by incubation with 2 % (v/v) H2SO4 in methanol(26). Fatty acid methyl esters were resolved using a HP6890 gas chromatograph (Agilent, Palo Alto, CA, USA) equipped with a BPX-70-fused silica capillary column (SGE, Melbourne, Australia), attached to an Orchid combustion interface (Europa Scientific Ltd, Crewe, UK) and isotopic enrichment was measured by a 20–20 stable isotope analyser (Europa Scientific Ltd). Plasma total TAG and NEFA concentrations were calculated from the GC-C-IRMS chromatograms by dividing the peak area for the fatty acids within the TAG and NEFA fractions by the peak area for the internal
Table 1. Characteristics of subjects

<table>
<thead>
<tr>
<th></th>
<th>Control (n 6)</th>
<th>noART (n 5)</th>
<th>NRTI (n 7)</th>
<th>NRTIPI (n 6)</th>
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<td>8.0</td>
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<td>17.7</td>
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<tr>
<td>Fat mass from BIA (kg)</td>
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<td>11.4</td>
<td>22.8</td>
<td>10.5</td>
</tr>
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<td>Lean body mass</td>
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<td>53.9</td>
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<td>Viral load (copies × 10⁻³/ml plasma)</td>
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<td>36.8</td>
<td>198.7</td>
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<td>CD4 count (cells × 10⁻⁹/l plasma)</td>
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<td>NA</td>
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<td></td>
<td></td>
<td></td>
<td>AZT 3TC (1)</td>
<td>D4T (1)</td>
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</table>

noART, not receiving antiretroviral therapy; NRTI, nucleoside reverse transcriptase inhibitor; NRTIPI, NRTI with protease inhibitors; K–W, Kruskal–Wallace; BIA, bioelectrical impedance analysis; D4T, stavudine; DDI, didanosine; AZT, zidovudine; 3TC, lamivudine; RTV, ritonavir; IND, indinavir; SQV, saquinavir; NFV, nelfinavir; NVP, nevirapine; ND, not determined; NA, not applicable.

Values are median (minimum and maximum) concentrations of glucose and insulin in plasma from control subjects and from HIV-positive patients who were not receiving noART, or were receiving NRTI or in combination with NRTIPI.

Statistical analysis for an effect of group was determined by K–W where significance assumed as P < 0.05.

ᵃᵇᶜ Values with different letters are significantly different by the Mann–Whitney U test (P < 0.05).
standards, and multiplying the result to account for the volume of plasma extracted and the amount of internal standard added. Enrichment of $^{13}$C in fatty acid methyl esters was expressed quantitatively as micrograms $[^{13}$C$]$palmitic acid/ml plasma\(^{(27)}\).

**Metabolic model and data analysis**

The primary changes of interest were the kinetics and time course of $[^{13}$C$]$palmitic acid in the TAG and NEFA pools within the circulation and the kinetics and time course of $^{13}$C-label excretion in breath. A summary statement of the TAG and NEFA excursions (both labelled and unlabelled) was derived by determining the AUC. Based on our earlier work\(^{(17–19)}\), prolonged retention of $[^{13}$C$]$palmitic acid as TAG in the circulation was assumed to reflect impaired chylomicron clearance from the circulation by peripheral tissues. Elevation of $[^{13}$C$]$palmitic acid in the NEFA fraction was assumed to reflect the hydrolysis of chylomicron TAG and impaired entrapment of the resultant fatty acids by peripheral tissues.

**Measurement of plasma glucose and insulin concentrations**

Plasma glucose concentration was determined using an automated glucose analyser (AU600, Olympic Diagnostics, Southall, UK). Plasma insulin concentration was measured using an automated ELISA assay (ES700: Roche Diagnostics, Lewes, UK).

**Statistical analysis**

Data were entered into a computerised database (SPSS version 10.0; SPSS Inc., Chicago, IL, USA). Given that the number of subjects in each group was relatively small and that much of the data were not normally distributed, the results are presented as median (range). An overall comparison between groups was carried out using Kruskall–Wallace analysis for multiple groups, and where a statistically significant group effect was observed, the Mann–Whitney \(U\) test was then used to determine the significance of the difference observed between any two groups. Statistical comparisons between groups of the postprandial response were restricted to comparisons of AUC. Statistical significance was assumed where \(P<0.05\).

**Results**

**Subject characteristics**

The groups were of comparable age and although the seropositive men tended to be lighter and exhibit lower BMI, triceps skinfold thickness, fat mass and lean body mass than the controls, these differences did not attain statistical significance (Table 1). Subjects in the noART group had significantly higher viral load and lower CD4 count than the other seropositive groups.

**Energy expenditure and substrate oxidation**

Measures of fasting and postprandial energy expenditure with the relative oxidation of macronutrients are shown in Table 2. In the fasting state, when expressed per unit lean body mass, REE was significantly greater in all three seropositive
groups relative to controls. There was no significant difference between groups in RER or net substrate oxidation in the fasting state.

In the fed state, energy expenditure expressed per unit lean body mass over 7 h was approximately 20–33 % greater in the seropositive groups compared with controls, with the greatest increases seen in the NRTI and NRTIPI groups (Table 2). There were no significant differences between groups in either the cumulative recovery of label on breath as $^{13}$CO$_2$ over 7 h expressed as a percentage of the administered dose of $[1-^{13}$C]palmitic acid or the relative proportions of carbohydrate and lipid (whether expressed as net, endogenous or exogenous lipid) oxidation to energy expenditure over the postprandial period (Table 2).

Postprandial changes in plasma concentration of TAG and $[^{13}$C]TAG, glucose and insulin

Fasting TAG was significantly elevated in all three groups of seropositive men than controls with modest elevations seen in noART and NRTI with much greater increases seen in the NRTIPI group (Table 1). Fasting NEFA concentration was raised in the NRTIPI group, but not the other seropositive groups, compared with controls. There were no significant differences between groups in fasting plasma glucose or insulin concentrations (Table 3).

Peak plasma metabolite concentrations in the postprandial state are shown in Table 3 and overall response to the meal is illustrated in Fig. 1 and summarised in Table 4. There was a significant effect ($P<0.05$) of subject group on peak plasma TAG concentration after the test meal. Peak TAG concentration was 2-8-fold higher in the NRTI group and 11-2-fold higher in the NRTIPI group compared with controls, while the noART group did not differ significantly from controls (Table 3). Peak TAG concentration was 3-9-fold higher in the NRTIPI group than the NRTI group (Table 3). There was a significant effect of group on plasma TAG AUC ($P<0.001$), on the incremental change in TAG AUC ($P<0.001$) and on AUC $[13$C]palmitic acid concentration in plasma TAG ($P<0.001$; Table 4).

Comparing the noART group with controls, there was no difference in TAG AUC or incremental change in TAG AUC. TAG AUC, but not incremental AUC, was significantly higher in the NRTI (3-1-fold) compared with controls (Table 4). For the NRTIPI subjects, TAG AUC and incremental AUC were significantly higher compared with controls (10-3- and 6-1-fold, respectively) and when compared with the NRTI group (3-3- and 3-6-fold, respectively; Table 4). The rank order for the overall plasma TAG response to the meal was control = noART $<$ NRTI $<$ NRTIPI. The AUC for $[13$C]palmitic acid in TAG did not differ significantly between controls and the noART group, while the NRTI group was 2-5-fold higher and the NRTIPI group 6-7-fold higher than controls (Table 4).

There was a significant effect of subject group on the extent of fall in plasma NEFA concentration after the meal (Table 3 and Fig. 1). The general pattern of fall and subsequent rise in NEFA concentration was similar across groups. Control, noART and NRTI groups started from comparable fasting NEFA levels and exhibited similar degrees of suppression as a percentage of baseline. Subjects in the NRTIPI group

| Table 3. Baseline and peak TAG, NEFA, glucose and insulin concentrations |
|-----------------------------|---------|----------|---|---|---|---|
|                           | noART   | NRTI     | NRTIPI |
| Baseline (n=6)             |         |          |       |
| TAG (mmol/l)               | 0.7 a   | 1.2 b    | 1.0 c  |
| NEFA (mmol/l)              | 0.26 a  | 0.16 a   | 0.28 b |
| Glucose (mmol/l)           | 5.1     | 6.2      | 5.6    |
| Insulin (pmol/l)           | 7.4     | 27.3     | 25.9   |
| Peak (n=8)                 |         |          |       |
| TAG (mmol/l)               | 1.28 a  | 1.25 b   | 1.21 c |
| NEFA (mmol/l)              | 0.11 a  | 0.10 a   | 0.16 b |
| Glucose (mmol/l)           | 8.1     | 6.2      | 6.1    |
| Insulin (pmol/l)           | 7.4     | 27.3     | 25.9   |

*Values are median (minimum and maximum) concentrations of glucose and insulin in plasma from control subjects and from HIV-positive patients who were noART or who were receiving without NRTI or in combination with NRTIPI.

fth values with different letters are significantly different by the Mann–Whitney U test ($P<0.05$).
exhibited a similar proportionate decrease but from a higher fasting baseline. In consequence, postprandial NEFA concentrations were similar to baseline concentrations in the other groups. The rank order for NEFA suppression was control; noART; NRTI; NRTIPI. There was no significant difference between groups in the AUC for \([13C]\)palmitic acid concentration in NEFA over 7 h (Table 4). There were no significant differences in glucose or insulin response between groups (Tables 3 and 4).

Discussion

Different classes of ART are associated with varying degrees of hypertriglyceridaemia in the fasted state. The results of the present study show that, in the postprandial state, NRTI therapy was associated with a raised and prolonged postprandial lipaemia that was further exacerbated by the addition of PI. These features were not seen in those seropositive men who were not on ART. The increased postprandial lipaemic response was associated with impaired delipidation of TAG-rich lipoproteins derived from lipid within the meal, as marked by the retention of label in the TAG fraction, most strikingly in those on ART containing both NRTI and PI. We were neither able to demonstrate any differences in entrapment of fatty acids released from TAG-rich lipoproteins after the meal as marked by accumulation of tracer in the NEFA fraction, nor was there any evidence of insulin resistance in the seropositive men on ART.

HIV infection \textit{per se} has previously been associated with demonstrable changes in fasting TAG and NEFA concentration \cite{28} and changes in energy expenditure \cite{29}. Previous studies in the fasted state report hepatic greater VLDL secretion in HIV-positive patients receiving PI-containing ART \cite{30}, which may be attributable to increased adipose tissue lipolysis \cite{31} and fatty acid re-esterification \cite{32}. We also found increases in fasting TAG and NEFA concentrations and REE, when expressed per unit lean body mass, consistent with chronic infection \cite{33}. However, since the NRTIPI group
had the lowest viral load, but had the highest fasting TAG, NEFA and REE, these findings cannot be attributed to chronic infection alone, but may well reflect more obvious and direct effects of the combining both NRTI and PI within a single ART regimen.

The magnitude of the incremental increase in plasma TAG following the test meal was significantly greater in the NRTI and NRTIPI groups compared with controls and reflected slower clearance of TAG derived from the meal and continuing presence of meal-derived lipid in the circulation. These observations are consistent with the delays in TAG-rich lipoprotein clearance observed in other studies of patients on PI-containing ART regimens\(^{20,21}\). Moreover, these findings suggest that NRTI treatment alone has a small, but significant, adverse effect on clearance of TAG from the meal, which is exacerbated when combined with PI therapy. There are two possible explanations for these findings, which are not mutually exclusive. Higher fasting TAG and impaired suppression of VLDL TAG secretion in the postprandial state\(^{15}\) may impair hydrolysis of meal-derived TAG as a result of increased competition between VLDL and chylomicron particles for lipoprotein lipase. Alternatively, lower lipoprotein lipase activity due to a direct effect of ART on synthesis\(^{32,34}\) or activation of the enzyme may impair TAG clearance. Taken together, both processes would result in slower removal of meal-derived lipid from the circulation. It is not possible to deduce the underlying mechanism from the present data.

It was not possible to directly measure the relative proportions of fatty acids released by the delipidation of chylomicrons that were taken up by the peripheral tissues in the present study, although some indication of the extent of entrapment can be derived from the accumulation of labelled fatty acid in the plasma NEFA pool. In this series, no obvious differences between groups were observed using this approach. It is important to note that the extent to which \(^{13}\)C-palmitic acid accumulates in plasma is not solely determined by the degree of entrapment, but would also be influenced by the availability of fatty acids from chylomicron-TAG hydrolysis, the uptake of fatty acids by the liver as well as any reappearance of fatty acids taken up by adipose tissue and then released back into the circulation through lipolysis. For example, marked reductions in the clearance of meal-derived TAG would mean that there would be less \(^{13}\)C-palmitic acid available to be taken up by peripheral tissues, and thus marked impairments in entrapment would yield comparable accumulation of \(^{13}\)C-palmitic acid in the NEFA pool. Thus, if less fatty acids were derived from TAG hydrolysis in the ART groups, then the same absolute accumulation of \(^{13}\)C-palmitic acid accumulation in the NEFA pool may reflect a much lower proportion of these fatty acids being taken up into the tissues (i.e. less entrapment). However, in the absence of modelling direct measures of flux across the tissue beds, we are not able to either confirm or refute the previous indirect reports of a possible effect of ART on fatty acid entrapment\(^{20,22,23}\).

In contrast to other reports\(^{3,15}\), the role of insulin resistance in impaired lipid metabolism in these subjects was less clear. Although there were no significant differences between groups, there were trends towards higher insulin and glucose concentrations particularly in the postprandial state. However,
in the NRTIPI group, the inability to suppress NEFA to the same concentration as seen in other groups would indicate that NEFA levels fail to fall below that usually seen in the fasted state. This may be due to a specific effect of NRTIPI therapy on the regulation of TAG hydrolysis by insulin on adipose tissue, or an effect of the drugs on other enzymes involved in the lipolytic pathway. This observation fits with the suggestion that higher levels of NEFA in the fed state contribute to the maintenance of an insulin-resistant state and serves to drive higher rates of VLDL–TAG synthesis and secretion(35).

It is important to recognise the limitations of these observations, most notably the small sample size and marked between-subject variance, particularly in the ART groups. The clinical phenotypes studied were typical of a patient group with a chronic infective disease receiving multiple therapies and, as such, it is to be expected that there would be heterogeneity among the subjects. We did not set out to impose further exclusion criteria to obtain discreet sub-populations of patients within the defined therapies in order to reduce variability so as to improve the statistical power of the study. To do so would further restrict the observations to a subgroup of the different treatment groups and may incur the criticism that the results were not typical of the overall patient group. It is equally important to recognise that the size of the study groups will always be limited by the size of the pool of potential patients willing to participate in complex, invasive metabolic studies of this kind. The number of subjects studied here is as large if not larger than many of the other published studies in this area of research and typical of metabolic studies in human subjects using stable isotope tracer technologies. Given these constraints, the possibility that the study was insufficiently powered given the heterogeneity to demonstrate differences in fatty acid oxidation or entrapment should not be excluded.

Overall, the present findings suggest that NRTI therapy induces dyslipidaemia at least in part by an impaired clearance of meal-derived TAG. The mechanisms that underlie this change in TAG-rich lipoprotein hydrolysis remain unclear. Equally, there is a need to determine the extent to which hypertriglyceridaemia in ART and other clinical states such as diabetes, metabolic syndrome, obesity and familial hypercholesterolaemia can be attributed to a failure to clear lipid and/or an increased VLDL–TAG secretion arising from perturbations in adipocyte TAG hydrolysis and increased NEFA flux to the liver or a failure to entrap the products of lipoprotein TAG hydrolysis and/or a failure to suppress VLDL secretion in the fed state. What is clear however is that we need a better understanding of the regulation of lipid metabolism in the fed state and how it might influence cardiometabolic risk. This point is of particular relevance to the treatment of HIV patients as recent evidence from the D.A.D cohort analysis has indicated that specific NRTI were associated with increased mortality from heart disease, which could not be attributed to fasting blood lipids(36). Therapies designed to prevent or minimise dyslipidaemia need to be considered that adverse effects of particular combinations of therapies may have varying metabolic effects in different tissues. From a nutritional perspective, if dyslipidaemia is to be minimised in ART, the specific adverse effects of particular combinations during the fed state should be considered and attention directed towards how such changes in metabolic regulation may be ameliorated or exacerbated by the types of meals consumed within the diet.

Acknowledgements


References