Macronutrient metabolism of adipose tissue at rest and during exercise: a methodological viewpoint

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The metabolism of white adipose tissue is regulated by many factors, including hormones and substrates delivered in the blood, the activity of the autonomic nervous system and the rate of flow of blood through the tissue. An integrated view of adipose tissue metabolism can only be gained, therefore, from studies in vivo. Of the various techniques available for studying adipose tissue metabolism in vivo, the measurement of arterio–venous differences offers some unique possibilities. In human subjects this technique has been performed mostly by catheterization of the venous drainage of the subcutaneous abdominal depot. Studies using this technique indicate that adipose tissue has an active pattern of metabolism, responding rapidly to meal ingestion by suppressing the release of non-esterified fatty acids, or to exercise with an increase in fat mobilization. Adipose tissue blood flow may also change rapidly in these situations; for instance, it increases markedly after a meal, potentially increasing the delivery of triacylglycerol to the enzyme lipoprotein lipase (EC 3.1.1.34) for hydrolysis. During exercise, there is evidence that adipose tissue blood flow does not increase sufficiently to allow delivery of all the fatty acids released into the systemic circulation. The various adipose tissue depots have their own characteristic metabolic properties, although in human subjects these are difficult to study with the arterio–venous difference technique. A combination of tracer infusion with selective catheterization allows measurements of leg, splanchnic and non-splanchnic upper-body fat mobilization and triacylglycerol clearance. Development of such techniques may open up new possibilities in the future for obtaining an integrated picture of adipose tissue function and its depot-specific variations.

Adipose tissue: Arterio–venous differences: Exercise

Even 5 years ago it was possible to hear an eminent biochemist stand up at a scientific meeting and describe white adipose tissue as rather inert from a metabolic point of view. That view has changed radically over the past few years. Partly, this change has come about because of the recognition of adipose tissue as an important secretory organ, producing not just leptin, a hormone with widespread effects on energy metabolism and other systems (Andrews, 1998), but a whole range of other peptides and biologically-active substances with both systemic and autocrine or paracrine effects (Flier, 1995; Sniderman & Cianflone, 1997; Gregoire et al., 1998). In addition, the plasticity of adipose tissue has been recognized through important new developments in understanding the regulation of adipose tissue gene transcription, and particularly those genes that control adipocyte differentiation and development (Guerre-Millo et al. 1996; Ailhaud, 1997; Gregoire et al. 1998). But even in the long-recognized area of macronutrient metabolism in white adipose tissue there have been developments. These developments have come about not least because of the development of new and improved techniques for studying this tissue in vivo, in human subjects and other animals (Arner & Bülow, 1993; Frayn et al. 1997b). A further development has been the recognition that adipose tissue blood flow (ATBF), long considered a potential regulator of metabolism in this tissue (Bülow & Madsen, 1981), is highly labile and responds rapidly to nutrient ingestion (Bülow et al. 1987; Samra et al. 1995; Summers et al., 1996). The present review will focus on macronutrient metabolism in white adipose tissue, and the methodology used to bring about new insights will be emphasized.

Abbreviations: ATBF, adipose tissue blood flow; LPL, lipoprotein lipase; NEFA, non-esterified fatty acids; TG, triacylglycerol.
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Studying adipose tissue metabolism: some methodological considerations

The study of adipose tissue metabolism reflects one of the triumphs of the reductionist approach. In the 1960s the American Physiological Society published the first comprehensive review of the metabolism of white adipose tissue (Renold & Cahill, 1965), in which a large proportion of the papers described work done with a particular fat depot of the rat, the epididymal fat pad. The latter preparation has many advantages, not least that it is readily incubated and its metabolism studied in vitro, and that it comes in pairs, so one fat pad can be used as a control while the contralateral pad from the same animal is used for testing different conditions. Rodbell (1964) published the first description of the preparation of isolated adipocytes, by collagenase digestion of the epididymal fat pad. Spherical adipocytes could now be studied in great detail, not least because cells from a number of depots could be pooled to create a uniform suspension, before distributing them amongst several flasks to test the effects of addition of hormones or substrates in different combinations or concentrations. In the past decade or so, adipose tissue has become a model tissue for studying precursor-cell differentiation using modern molecular biological techniques (Guerre-Millo et al. 1996; Ailhaud, 1997; Gregoire et al. 1998).

However, these increasingly refined methods for studying adipose tissue have tended more and more to distract from the important concept that adipose tissue is more than just a collection of adipocytes. It is a highly structured tissue in which vascularization, innervation and cellular arrangement are all important. Some critical metabolic events in adipose tissue take place, not even within or on the surface of the adipocyte, but in the extracellular environment. A clear example is the action of lipoprotein lipase (EC 3.1.1.34; LPL), the enzyme responsible for hydrolysis of triacylglycerol (TG) in the circulating TG-rich lipoprotein particles (chylomicrons and VLDL), which makes fatty acids available for uptake by adipocytes. LPL, although synthesized within adipocytes, is secreted and moves to its site of action, the capillary lumen, where it attaches to endothelial cells via proteoglycan chains and can therefore come into contact with the TG-rich lipoprotein particles, which are largely confined to the vascular space.

It is therefore essential that, alongside these elegant techniques for looking in finer and finer detail at molecular aspects of the adipocyte, we do not lose sight of the need for integrative methods of looking at adipose tissue, which will reflect the complex events governing adipose tissue metabolism in vivo. I have suggested previously that these methods may be grouped under three headings (Frayn et al. 1997b):

1. Systemic tracer techniques (e.g. measurement of the plasma non-esterified fatty acid (NEFA) production rate, which reflects whole-body NEFA release from adipocytes);
2. Microdialysis, enabling the concentrations of (mainly water-soluble) molecules in the interstitial fluid to be measured;
3. Arterio–venous difference methods, enabling quantitative estimates of the flux of metabolites (including NEFA and TG) to be made, but posing fairly severe anatomical restrictions.

Microdialysis methods have been covered thoroughly in the present symposium by Arner (1999), Bangsbo (1999) and Henriksson (1999). Here I will concentrate on arterio–venous difference measurements. These methods have been reviewed previously (Frayn, 1992; Frayn et al. 1993), but the present review will update those earlier ones. The aim of the following review is to present a picture of adipose tissue metabolism largely, but not exclusively, gained using arterio–venous difference measurements, and with an emphasis on the methodology used.

Outline of macronutrient metabolism in white adipose tissue in relation to the whole body

White adipose tissue plays an important role in whole-body macronutrient metabolism. This is obvious in view of its unique position as the only tissue able to release NEFA into the circulation. However, the fatty acids that adipose tissue releases have, for the most part, previously been taken up from the circulating TG-rich lipoproteins: chylomicrons (carrying dietary fat) and VLDL (carrying TG secreted from the liver). It is not surprising therefore that adipose tissue also has an important place in the clearance of TG from the plasma, through the activity of the enzyme LPL. The total flux of fatty acids in and out of adipose tissue is difficult to quantify, but the daily flux (in energy equivalents) may be approximately 5 MJ/d in normal human subjects (Frayn et al. 1995b). Fatty acid flux is substantial in terms of whole-body energy flux, showing that TG and fatty acid metabolism in white adipose tissue are of major importance in whole-body macronutrient metabolism.

NEFA and TG metabolism interact closely in adipose tissue. The action of LPL on the TG-rich lipoproteins generates fatty acids, as does intracellular lipolysis, and the net movement of fatty acids (into adipocytes for esterification and storage in the postprandial state; out of adipocytes for export to the rest of the body during fasting or exercise) is governed by coordinated regulation of the enzymes LPL and hormone-sensitive lipase (EC 3.1.1.3), together with the pathway of fatty acid uptake and esterification. Whilst the whole-body release of NEFA can be studied by measurements of systemic NEFA production rates using isotopic tracers, the greater level of detail that can be uncovered using arterio–venous difference measurements has enabled this picture of fatty acid metabolism to be built up (Frayn et al. 1995a).

Glucose uptake by adipocytes has been studied in detail in isolated or cultured adipocytes in vitro. It is insulin sensitive and is brought about by the insulin-regulated glucose transporter type 4 (GLUT4) with a contribution from glucose transporter type 1 (GLUT 1). However, in vitro studies do not give useful quantitative information on the importance of this process to whole-body glucose utilization. Measurements made by two separate methods in vivo show, however, that glucose uptake by white adipose tissue is not a major pathway in terms of whole-body metabolism. These estimates have been obtained both by arterio–venous difference measurements and also by a
combination of tracer and biopsy techniques, and they suggest that in lean individuals adipose tissue is responsible for disposal of approximately 4% (maximum 10%) of an oral glucose load (Márin et al. 1987; Coppack et al. 1990), compared with 30–35% into skeletal muscle (Coppack et al. 1990; Taylor et al. 1993).

Of the glucose that is taken up, a proportion is oxidized, a proportion released as lactate (and probably as alanine) and a proportion used for synthesis of glycerol-3-phosphate, a proportion released as lactate (and probably as alanine) and a substantial contribution of anaerobic glycolysis (Márin et al. 1987; Newby et al. 1988). Estimates in vivo are dependent on the methodology. Arterio–venous difference measurements suggest a much smaller contribution, typically approximately 15–30% of the glucose taken up is released as lactate (Frayn et al. 1989; Coppack et al. 1990) and a correspondingly small contribution to whole-body lactate production (1% or less of an oral carbohydrate load is needed for esterification of fatty acids taken up from the action of LPL. Studies of isolated adipocytes suggest a much smaller contribution, typically approximately 15–30% of the glucose taken up is released as lactate by adipose tissue; Frayn et al. 1989; Coppack et al. 1990, 1996). However, measurements made by microdialysis suggest much greater lactate production, that may be significant in whole-body terms (Jansson et al. 1994; Simonsen et al. 1994). Estimates of lactate production by the two techniques therefore show a large discrepancy, the reason for which is not understood (Simonsen et al. 1994).

Amino acid metabolism in white adipose tissue is not well understood, although a number of studies both in vitro and in vivo suggest that it is relatively active. In vivo studies have used both microdialysis (Kowalski & Watford, 1994) and arterio–venous difference measurements (Frayn et al. 1991; Kowalski et al. 1997), and show glutamine release from adipose tissue, along with alanine, at rates that suggest a major role for adipose tissue in whole-body fluxes of these amino acids, and in that of glutamate. Interestingly, some studies of adipocytes in vitro show net utilization of glutamine (Kowalchuk et al. 1988). The reason for the discrepancy is not clear.

A brief description has been given of the major features of white adipose tissue macronutrient metabolism. This alone should be sufficient to counter any description of white adipose tissue as relatively inert in metabolic terms. The complexity of white adipose tissue metabolism in vivo as revealed by arterio–venous difference measurements is illustrated in Fig. 1.

However, the metabolic importance of white adipose tissue is most clearly seen by considering the dynamic changes that occur in response to physiological situations such as fasting and feeding, and exercise.

**Macronutrient metabolism of white adipose tissue in fasting and fed states**

In the post-absorptive state fatty acids are a major source of oxidative fuel in the body. These fatty acids arise largely from white adipose tissue, exported in the form of NEFA. Some tissues oxidize fatty acids taken up from circulating VLDL particles by the action of LPL. However, these fatty acids will themselves have come largely from adipose tissue, taken up by the liver and re-esterified into TG for secretion. Thus, in the post-absorptive state white adipose tissue controls the flux of a major oxidative fuel to other tissues in the body.

After feeding, this situation changes rapidly. In the case of a mixed meal containing carbohydrate and fat, exogenous glucose enters the plasma and there is a rapid switch in whole-body fuel economy, so that glucose displaces fatty acids as the major fuel for many tissues, including skeletal muscle (Elia et al. 1988). It is well established that elevated concentrations of fatty acids may impair insulin stimulation of glucose utilization by skeletal and cardiac muscle (Randle et al. 1963; Ferrannini et al. 1983), and therefore it is appropriate that NEFA release from adipose tissue is extremely sensitive to suppression by insulin (Taylor et al. 1984; Jensen et al. 1989; Campbell et al. 1992). In fact NEFA release from adipose tissue changes from its maximal rate in the typical 24 h cycle to approximately zero within 60–90 min of eating a mixed meal (Coppack et al. 1990, 1992; Fig. 2). At the same time, other processes are stimulated to reverse the net release of fatty acids from adipocytes and begin net uptake of fatty acids for esterification and storage. These processes include the up regulation of LPL by insulin (Sadur & Eckel, 1982; Ong & Kern, 1989) and perhaps by gut hormones (Oben et al. 1992). However, possibly the most important change is the stimulation of fatty acid esterification in adipocytes, brought about by insulin (Leboeuf, 1965; Frayn et al. 1994) and by acylation-stimulating protein (Sniderman & Cianflone, 1997; Sniderman et al. 1997). Aciylation-stimulating protein is a peptide, identical to C3a-desarg, a product of the alternative complement pathway, that is produced locally in adipose tissue and feeds back to stimulate fatty acid esterification. Its production is stimulated by the presence of chylomicrons (carrying dietary fat), thus leading to close coordination of metabolic events in adipose tissue in the postprandial period (Maslowska et al. 1997).
Measurement of arterio–venous differences has allowed the construction of this dynamic view of the regulation of adipose tissue metabolism in the post-absorptive–postprandial transition. Tracer methods for measuring NEFA production rates can give useful information in the non-steady-state such as that following meal ingestion (Jensen et al., 1990), but measurement of arterio–venous differences for NEFA, TG and glycerol has enabled the integration of NEFA and TG metabolism to be studied during this phase (Frayn et al., 1995b). The secretion of acylation-stimulating protein by adipose tissue, coordinated with postprandial TG metabolism, has also been demonstrated using this technique (Saleh et al., 1998). The use of arterio–venous difference measurements in the non-steady-state may itself pose problems (Zierler, 1961), but it has been argued that these problems may not be as severe as sometimes claimed (Elia et al., 1988).

**Potential role of blood flow as a regulator of white adipose tissue metabolism**

It will be clear from the earlier discussion that the major metabolic exchanges between adipose tissue and blood are of the hydrophobic molecules NEFA (bound to albumin), and TG carried in the TG-rich lipoproteins. These substances, unlike small hydrophilic molecules such as glucose and glycerol, cannot diffuse readily through interstitial fluid. It is not surprising therefore that white adipose tissue is relatively well vascularized; the number of capillaries per unit cytoplasm is typically greater than in skeletal muscle (Frayn & Macdonald, 1996). Furthermore, ATBF is highly variable according to physiological state (Frayn & Macdonald, 1996; Frayn et al., 1997a). The close connections between adipose tissue metabolism and ATBF have been reviewed previously (Crandall & DiGirolamo, 1990; Frayn & Macdonald, 1996; Frayn et al., 1997a). In brief, it seems that ATBF always increases in parallel with stimulation of fat mobilization. This relationship is intuitively reasonable since increased ATBF is needed to transport the product of lipolysis (NEFA) away from the tissue. The coordination of ATBF and stimulation of lipolysis (measured using arterio–venous differences) is illustrated in Fig. 3. More recently we have come to appreciate that ATBF also increases in the postprandial period (Coppack et al., 1992; Samra et al., 1995; Summers et al., 1996), and we have argued (Frayn et al., 1997a) that this increase may represent the need to deliver substrate (TG in the chylomicrons) to adipose tissue LPL for rapid clearance. Indeed, in an experiment in which ATBF was increased by infusion of adrenaline, local TG clearance measured using arterio–venous differences increased in parallel with ATBF, suggesting that it may normally be flow limited (Samra et al., 1996). The potential role of ATBF in limiting metabolism will be further discussed in connection with exercise (see pp. 881–882).

**Regional variations in adipose tissue macronutrient metabolism**

It has become clear in recent years that the various discrete adipose depots have their individual metabolic characteristics. These observations have been prompted by the association between fat distribution, insulin resistance and cardiovascular risk factors, with an excess accumulation of fat in the upper body or abdominal region associated with increased risk of development of type 2 diabetes and of CHD (Björntorp, 1991). A brief summary is that the lower-body (gluteal and femoral) adipose depots are characterized by a relatively sluggish pattern of metabolism, responding less than other depots to lipolytic stimulation (Arner et al., 1990). The subcutaneous abdominal adipose tissue has an intermediate metabolic activity, and intra-abdominal depots have the most active pattern of metabolism (Rebuffe-Scrive et al., 1987, 1989, 1990; Richelsen et al., 1991; Arner, 1995). The mechanisms involve differences in receptor density and function (sensitivity to stimulation) in the different depots (Vikman et al., 1991; Arner, 1995; Hofstedt et al., 1997).
The receptors involved include adrenergic β-receptors stimulating lipolysis, and insulin and adenosine receptors suppressing it. The hypothesis has been advanced that the intra-abdominal depots, some of which discharge the products of lipolysis directly into the portal vein, have direct effects on hepatic metabolism. In particular, it is envisaged that a high flux of NEFA from omental adipose tissue will drive VLDL-TG secretion, stimulate hepatic glucose production and potentially decrease hepatic insulin clearance (Björntorp, 1994; Arner, 1997), the so-called ‘portal theory’ (Arner, 1997).

It is wise to remember, however, that these observations are to a large extent based on studies of isolated explants of adipose tissue or isolated adipocytes, and caution must be exercised, as discussed earlier, before extrapolating such observations to the situation in vivo. Microdialysis can be used to sample interstitial glycerol concentrations in different subcutaneous depots (although not intra-abdominal depots) and such studies confirm the greater metabolic lability of abdominal v. gluteal fat (Arner et al. 1990). Arterio–venous difference measurements should provide an ideal method for confirmation, or otherwise, of these observations in vivo. However, they are difficult to perform for anatomical reasons. Confirmation of the portal theory really requires sampling from the portal vein, which is difficult in human subjects, although it has been performed in a small number of studies (usually at laparotomy). The results of these studies have been reviewed in more detail by Frayn et al. (1997c), but they do not generally confirm the idea of a high concentration of NEFA (or glycerol) in the portal vein compared with other sites.

An alternative approach is to use combined tracer infusion and arterio–hepatic venous sampling to assess arterio–venous differences across the splanchnic bed. If it is assumed that the liver does not release glycerol or NEFA, then any dilution of labelled fatty acids or glycerol observed across the splanchnic bed may be presumed to reflect lipolysis in splanchnic adipose tissue. Such studies, using either labelled fatty acids or glycerol, do show some dilution of label, but the rate of lipolysis in splanchnic adipose tissue estimated in this way appears small in relation to that in the whole body. Nevertheless, it must be remembered that the products of this lipolytic process are delivered directly to the liver and so, as discussed earlier, may have a metabolic impact greater than their absolute amount might suggest.

Recent studies from Jensen and colleagues (Jensen & Johnson, 1996; Nguyen et al. 1996) have brought new methodology to bear on this issue. They combine selective catherization of the femoral and hepatic veins, providing information on lower-body (leg) and splanchnic bed fat metabolism. Concomitant infusion of a radio-labelled fatty acid tracer allows the calculation of whole-body, leg and splanchnic rates of release and uptake of fatty acids. Splanchnic NEFA release is assumed to arise from intra-abdominal adipose tissue. By subtraction, the rate of NEFA concentration in the venous drainage from subcutaneous adipose tissue during exercise (Arner et al. 1993). However, the systemic plasma NEFA concentration may not rise as much as the rate of appearance, leading to dissociation of the normal close relationship between NEFA production rate and systemic NEFA concentration. NEFA concentrations in the venous drainage from subcutaneous adipose tissue increase much more than those in systemic plasma, implying that there is rapid utilization, presumably by the working muscle (Hodgetts et al. 1991). NEFA released from adipose tissue may be oxidized in other tissues, or they may be re-esterified. This re-esterification may be local within the same depot (perhaps even within the adipocyte) or it may occur in other tissues.
The whole-body re-esterification of fatty acids falls dramatically during exercise (Wolfe et al. 1990), so leaving more fatty acids for oxidation. This situation is also true within adipose tissue; the proportion of fatty acids re-esterified within adipose tissue (estimated using arterio–venous difference measurements of NEFA:glycerol release) decreases to approximately zero during 1 h of exercise at 50–70 % of maximum \( O_2 \) uptake (Hodgetts et al. 1991). However, these calculations may be misleading (see discussion below); the fatty acids retained in adipose tissue may be retained in a non-esterified form.

ATBF usually increases when lipolysis is increased, as discussed previously. Since \( \beta \)-adrenergic stimulation itself has vasodilator effects in adipose tissue (Hjemdahl & Linde, 1983; Simonsen et al. 1990), it might be expected that ATBF would increase during exercise. In fact there are few findings to suggest this increase occurs. Bülow & Madsen (1976, 1978) showed large increases in ATBF during prolonged moderate-intensity exercise (50 % maximum \( O_2 \) uptake), manifest during the first hour but increasing substantially with further 50 min periods of exercise (Fig. 4). Other studies, however, have failed to show this increase in ATBF (Frid et al. 1990; Hellström et al. 1996). It could be argued that in heavy exercise so much of cardiac output is diverted to the working muscles that there is little left to increase ATBF. This situation would parallel the fall in splanchnic blood flow during strenuous exercise, which may limit the contribution of hepatic gluconeogenesis to substrate supply (Fojt et al. 1976). Even in the study of Bülow & Madsen (1976) it can be argued that ATBF did not increase to match the increased need for delivery of NEFA to the circulation (Fig. 4). The validity of this argument becomes very clear when rates of NEFA release are examined at different exercise intensities; the rate of appearance of NEFA actually falls as exercise intensity increases from light to heavy (Jones et al. 1980; Romijn et al. 1993). Since the stimuli to lipolysis are all likely to increase in relation to the intensity of exercise, this finding presumably reflects insufficiency of ATBF to deliver all the NEFA that might be released in lipolysis into the circulation. In fact, during heavy exercise, the relative rates of appearance of glycerol and NEFA show that lipolysis occurs but does not lead to release of NEFA into the plasma (Jones et al. 1980). This view is confirmed by the sudden release of NEFA, without accompanying glycerol, at the end of exercise (Hodgetts et al. 1991; Romijn et al. 1993). However, arterio–venous difference measurements across subcutaneous adipose tissue have not been performed at different intensities of exercise, and so the potential of this technique to demonstrate more clearly the entrapment of fatty acids in adipose tissue during high-intensity exercise has not yet been exploited. In part this situation reflects the technical difficulties of these measurements.

Critical review of methodology

The arterio–venous difference technique for studying macronutrient metabolism in adipose tissue has allowed a dynamic picture of adipose tissue metabolism and its regulation in different physiological states to be built up. The technique has been applied in a number of species, including dog (Bülow et al. 1985; Holloway et al. 1985), fat-tailed sheep (Gooden et al. 1986), rat (Kowalski et al. 1997) and human subjects (Frayn et al. 1989). This technique allows the quantitative estimation of net substrate fluxes. When combined with tracer infusions this technique may allow calculations of absolute uptake and release rates (Kurpad et al. 1994). In many cases qualitatively similar information is gained from microdialysis and arterio–venous difference techniques (Simonsen et al. 1994; Kowalski et al. 1997; Summers et al. 1998), but quantitative estimates of substrate flux do not always agree as well (Simonsen et al. 1994; Summers et al. 1998).

Some limitations of the arterio–venous difference technique must be borne in mind. First, because of anatomical limitations, its use in human subjects is probably restricted to the anterior abdominal subcutaneous adipose depot. There is evidence that in terms of NEFA release this depot behaves typically of all the adipose tissue that contributes to the systemic plasma NEFA concentration (Frayn et al. 1993). Second, catherization of the venous drainage of adipose tissue is technically difficult, and even with experience success rates never exceed about 80 %. Some experiments are therefore wasted, especially in experimental designs that require subjects to return on more than one occasion. There is limited experience of using this technique during exercise. Our own experience shows that this is possible, but again technically difficult (Hodgetts et al. 1991). It has not been used at intensities greater than 70 % maximum \( O_2 \) uptake and our limited experience suggests this would be very difficult (but not necessarily impossible).

An alternative approach, combining tracer methods for measuring absolute rates of NEFA uptake and release with selective catherization, has recently been developed. This...
approach allows assessment of regional NEFA production and utilization rates (leg, upper-body non-splanchnic and splanchnic; Jensen & Johnson, 1996; Nguyen \textit{et al.}, 1996), and is a useful complement to the anatomically-stricter catheterization of subcutaneous abdominal adipose tissue venous drainage.

\textbf{Conclusion}

White adipose tissue is an important site of macronutrient metabolism, both in its own right, and as the supplier of NEFA to other tissues. We now recognize white adipose tissue to have an extremely active and highly regulated pattern of metabolism, which complements its role as a secretory organ, particularly in relation to the production of leptin which itself is a major controller of macronutrient metabolism. New techniques have made the study of adipose tissue metabolism \textit{in vivo} feasible, and thus helped to highlight its metabolic importance, especially the techniques of microdialysis and arterio–venous difference measurement. The regulation of ATBF is intimately linked with macronutrient metabolism in adipose tissue.

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\textbf{References}


