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Some New Insights into Biology and Medicine through NMR Spectroscopy

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Some new insights into biology and medicine through NMR spectroscopy

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The contributions to biology and medicine by NMR spectroscopy *in vivo* require careful definition of the problems that are studied. Temporal and spatial resolution of the biochemical information obtained are the key to success, although the latter is limited owing to low sensitivity and small concentrations of the metabolites studied. Using ^{31}P NMR investigations in four areas are described. Control of energetics by ADP in normal and diseased muscle is shown to be important. Enzyme catalysed fluxes are obtained for creatine kinase and ATP synthase in muscle and in the human brain enzyme activity maps are derived. The measurements on the ionic environment and fluxes for H^+, Na^+ and K^+ (Rb^+) give us new information about the role of ions in cell proliferation (e.g. in cancer) and hypertension. Molecular architecture of phospholipids *in vivo* is readily observed and is perturbed in the brain in chronic head injury and demyelination.

1. Introduction

At about the same time as the first experiments on NMR imaging were carried out, quite independently biochemical laboratories have demonstrated that high-resolution spectra, particularly from ^{31}P, can be obtained from intact tissue samples (Hoult *et al.* 1974). Human studies became possible in the 1980s with the introduction of surface coils and high field magnets, initially suitable for human limbs but later, in about 1983 for whole body. In our case, a combined biochemical and clinical approach has been critical for asking specific questions about human disease and the underlying biochemical mechanisms. There are two important aspects to the approach we adopted in clinical work. In the first, we are concerned with temporal resolution in response to a stress or some kind of intervention. This is exemplified best in our extensive series of investigations on normal and diseased human muscle which now includes the study of over 1000 different cases (Radda *et al.* 1989). The second approach requires spatial resolution in as much as that is possible with the limited sensitivity of the nuclei and concentrations of substances one is observing. Two of the following papers will discuss some of the developments in improving spatial resolution and Dr Peter Styles will present at the Ciba Symposium our own evaluation of the method which we use in a fairly routine way in our clinical studies. We have now carried out a large number of investigations of the human brain, liver and heart using the technique of phase-modulated rotating frame imaging in which we can get spectra from slices of *ca.* 10–25 ml of tissue and obtain one-dimensional

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maps of the phosphorus-containing metabolites and of intracellular pH (Blackledge *et al.* 1987). We have also been able to use this method to relate the localization of the spectra to surface coil proton images and to get absolute concentrations for the observed metabolites (Cadoux-Hudson *et al.* 1990*c*). In this presentation I shall address four areas where I believe spectroscopy has already made significant contributions both to biochemical and clinical investigations. These are concerned with the control of pathways, with fluxes of intracellular reactions, with the transport and control of ionic concentrations and finally with macromolecular assembly and macromolecular structure *in vivo*.

2. Control *in vivo*

We had investigated the energetics of human organs and tissues by observing the pathways which are well known and include oxidative synthesis of ATP, the reaction catalysed by creatine kinase, and the glycolytic sequence. We can measure the concentrations of many of the phosphorus containing metabolites in these pathways and directly obtain the activity *in vivo* of the enzyme creatine kinase and that of ATP-synthase. Under some conditions we can measure the flux through glycolysis and the role of hydrogen ions in the control of these various processes.

In the first instance I would like to demonstrate the importance of ADP as a regulator in muscle and how this control is intimately linked to the reaction catalysed by creatine kinase (figure 1). ADP is now known to be, at least in skeletal muscle, a positive effector of oxidative phosphorylation and an inhibitor of contractility. In resting muscle its concentration as determined from the creatine kinase equilibrium, is between 5 and 10 μM. When the work is increased, the rate of oxidative phosphorylation needs to be stimulated and this is achieved by allowing ADP to increase in concentration. It is expected that the K_m for the ADP control of mitrochondrial ATP synthesis is about 25 μM, so with increased demand for energy as long ADP increases up to *ca.* 70–100 μM oxidation can be maximally stimulated. If on the other hand ADP is allowed to increase above that level it will begin to inhibit contractility. We have proposed the hypothesis, and since then demonstrated it to be true in a number of cases, that the concentration of ADP is strictly controlled within the limits that are desirable for its positive effect on oxidation and undesirable for its negative effect on contractility (Radda 1986). Creatine kinase has the role of controlling the concentration of ADP through the equilibrium described by the equations (in figure 1). Hydrogen ions are also important in this equilibrium. When ADP increases too much, glycolysis is turned on, lactic acid is produced, intracellular pH drops and this way the concentration of ADP is maintained or suppressed to get below the inhibitory level for contraction.

This mechanism of control has implications in a number of clinical conditions and I wish to show you one example, that of muscle contraction in patients with myotonic dystrophy. This is a condition that results in the inability of the patient to relax its muscle after a hand grip, such as shaking hands, and the underlying cause, or for that matter in this case the genetic background to this cause is still largely unknown. When such patients are asked to carry out our standard dynamic exercise (Taylor *et al.* 1983) and the breakdown of phosphocreatine and change in intracellular pH are followed, we find that the relation between pH and PCr breakdown is abnormal as shown in figure 2. Although there is a significant decrease in

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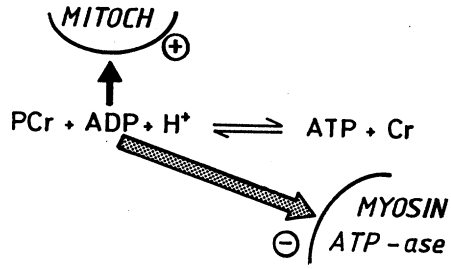

Figure 1. Control by ADP and creatine kinase. Free ADP is *ca.* 5–10 μM.

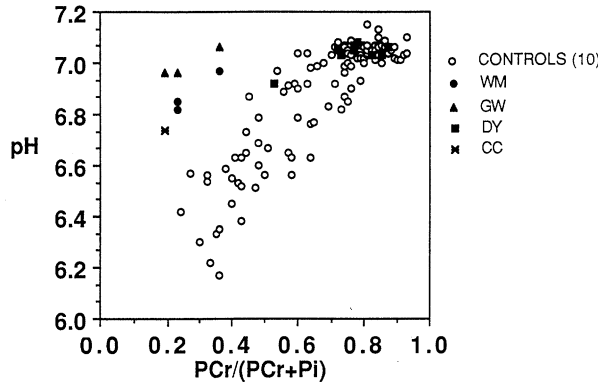

Figure 2. Change in pH and PCr in forearm muscle measured by ^{31}P MRS with increasing exercise in patients with myotonic dystrophy and controls (D. J. Taylor, G. Kemp, J. H. Edwards & G. K. Radda, unpublished results).

phosphocreatine, there is relatively little acidification of the muscle in the patient group. This means that unlike in normal controls where ADP increases during dynamic work to 40–70 μM, patients with myotonic dystrophy give a very rapid and substantial rise in ADP because as I explained before, there is no compensatory suppression through the creatine kinase equilibrium by hydrogen ions produced in glycolysis. We believe that just like in patients with McArdle's Syndrome (Radda 1986) this increase in ADP concentration could account for the physiological expression of their genetic abnormality, namely the inability to relax the muscle or alternatively the decrease in muscle output.

3. Reaction fluxes *in vivo*

In many cases new ideas and hypotheses have been derived from studies on patients and then tested in detail in appropriate animal experiments. Here I briefly outline our studies on the control by ADP on the rate of ATP synthesis *in vivo*. We used ^{31}P NMR magnetization transfer measurements to measure the flux between phosphocreatine and ADP, and ATP and inorganic phosphate during steady-state isometric muscle contraction in the rat hind limb *in vivo* (Brindle *et al.* 1989). Steady-state contraction was obtained by supramaximal sciatic nerve stimulation. Increasing the stimulation pulse width from 10 to 90 ms at a pulse frequency of 1 Hz

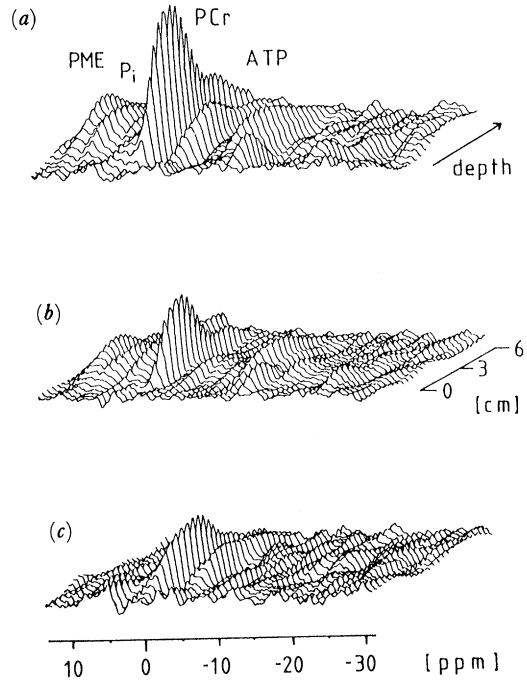

Figure 3. Stack plots of PMRFI images of human brain with (*a*) control irradiation; (*b*) irradiation of the γ-ATP; (*c*) the substraction of (*b*) and (*a*). Distance is shown on the γ-axis and chemical shift (from PCr) on the *x*-axis. The two data-sets consisted of 2×10 increments of four scans at a repetition delay of 15 s for both (*a*) and (*b*). The total acquisition time was therefore approximately 40 min. (Cadoux-Hudson *et al.* 1989*a*.)

or increasing the frequency of a 10 ms pulse from 0.5 to 2 Hz did not effect the flux through creatine kinase, suggesting that this enzyme is not acting as a shuttle for phosphocreatine between the mitochondria and cytoplasm as has been proposed (Bessman & Carpenter 1985). At the same time the ATP synthase flux increased, dependent on the concentration of 'free' ADP. The data were consistent with the control of mitrocondrial ATP synthesis by cytosolic ADP and indicated an apparent K_m of the mitrocondria for ADP of around 30 μM, consistent with the hypothesis I proposed earlier.

The same kind of fluxes can be measured in humans (Rees *et al.* 1988) and in the brain can even be resolved spatially using a combination of the rotating frame experiment with the saturation transfer measurements (Cadoux-Hudson *et al.* 1989*a*). We have carried out a detailed study of the creatine kinase activity as a function of its location within the human brain and have shown that while the distribution of the phosphorus-containing metabolites (i.e. phosphocreatine, ATP and P_i), as well as of pH is independent of depth from which the slice is taken, i.e. there is no difference between grey and white matter, the activity of creatine kinase is a factor of two smaller in the white matter than in the grey matter. Figure 3 gives, if you like, an 'enzyme activity image' across the human brain.

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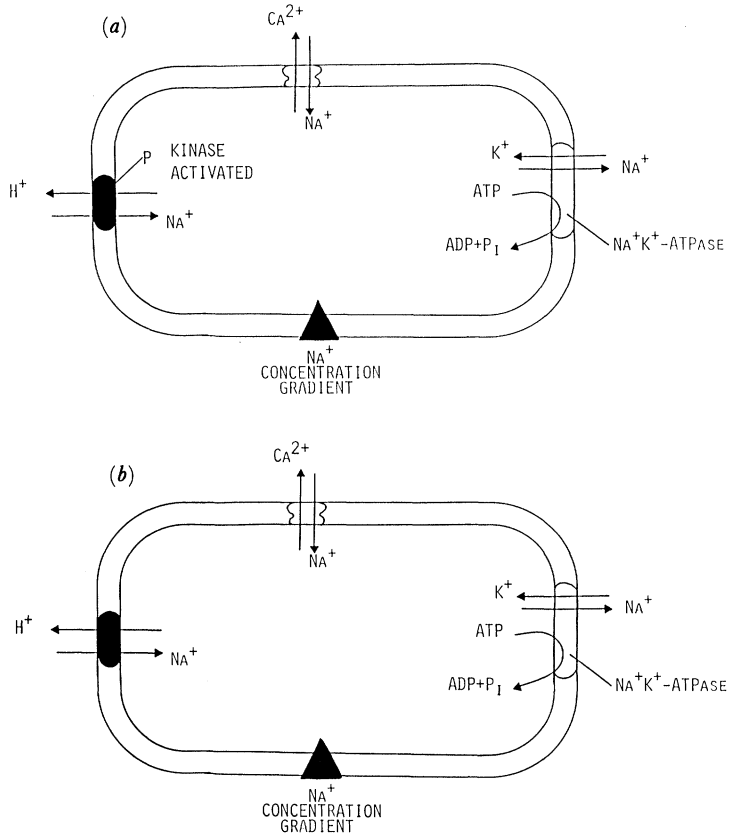

Figure 4. Cellular ion fluxes and control. (*a*) Dividing cell; (*b*) ion movement in cells.

4. Ion fluxes and ionic control

Intracellular ionic homeostasis is important in cellular functions. The main mechanisms by which this is achieved are demonstrated in figure 4. The important constituents in that are the ATP linked sodium/potassium ATPase, the sodium potassium exchange, the hydrogen–sodium antiport and various channels for calcium movement. In addition to these processes there are intracellular stores for calcium. Calcium is an important ion in the control of a variety of intracellular processes. H^+ is also thought to be involved in several processes, for example possibly as a trigger for protein synthesis during hypertrophy and during mitogenic stimulation. Indeed it has been proposed that intracellular hydrogen ion is pumped out at an increasing activity during cell division as the sodium–hydrogen exchange protein in the plasma membrane is phosphorylated by a phosphorylase kinase C which in turn depends on the control mechanisms associated with some of the lipid signalling pathway (for a brief review see Nishizuka 1983). We have shown some years ago that in human tumours, particularly in the brain, the intracellular pH is alkaline contrary to what has been expected from earlier theories about lactic acid production by glycolysis in tumours (Oberhaensli *et al.* 1986). We proposed that this alkalinization may well represent a stimulation of the sodium–hydrogen exchange during tumour growth. Since then we have extended these observations to over 40 brain tumours (Cadoux-Hudson *et al.* 1989*b*) and our results have also been

Table 1. *Metabolic changes at tumour centre compared with normal brain*
(Significance by Wilcoxon 2-sample, non-parametric, test.)

	normal ($n = 5$)	glioma ($n = 7$)	menangioma ($n = 4$)
pH_i	7.03 ± 0.02	7.08 ± 0.03[a]	7.19 ± 0.02[a]
range	6.96–7.06	7.02–7.11	7.15–7.28
PCr/ATP	1.03 ± 0.22	2.09 ± 0.5[a]	0.64 ± 0.15[a]
range	0.8–1.3	1.01–3.3	0.34–0.81
PME/ATP	0.65 ± 0.2	1.16 ± 0.4[a]	1.08 ± 0.05[a]
range	0.42–0.91	0.82–1.78	1.02–1.12

[a] $p = < 0.5\%$.

confirmed by from many other laboratories. In table 1 I summarize some of our results on gliomas and menangiomas showing the changes in the phosphocreatine to ATP ratio, in the ratio of the phosphomonoester to ATP and in intracellular pH. If the increase in pH is to do with growth factor stimulation, then we might predict that under certain conditions, where such factors are at high levels, we should observe similar changes. We have therefore studied a group of patients with the condition known as acromegaly, in which a pituitary adenoma leads to increased growth hormone secretion which in turn produces increased concentrations of growth factors. Six patients with pituitary adenomas were studied before and three days after transphenoidal surgery. Three patients were secreting large quantities of growth hormone and one had moderately raised levels. The temporoparietal ^{31}P image was used to investigate the metabolism of cells near the pituitary adenoma, but not in the tumour. Three of these four patients had elevated pH_i in deep white matter (*ca.* 5 cm depth), possibly indicating that normal cells respond to a factor released from the adenoma by a rise in pH_i (Cadoux-Hudson 1990). This is consistent with the hypothesis that I proposed above. There are, of course, other explanations for such changes in pH_i as besides sodium hydrogen exchange the CO_2 bicarbonate equilibrium has an important role in the control of intracellular hydrogen ion concentration. In other conditions, such as muscular dystrophy, we also observe increases in pH_i which are likely to be associated with abnormal intracellular calcium concentrations. To study the former effect, i.e. the CO_2 bicarbonate equilibrium, on brain intracellular pH we investigated the spatially resolved signals from a group of volunteers breathing normal air and increased CO_2, namely 5% CO_2, with 95% oxygen. Hypercapnia increases the end-tidal CO_2 and shows increased blood levels of CO_2. Intracellular pH in the normal human brain is about 7.03 and it shows no variation with depth, while in hypercapnia we can see a significant acidification to a value of about 6.92 as we reach deeper into the white matter (Cadoux-Hudson *et al.* 1990*b*). The possible reasons for this depth dependence have been discussed elsewhere.

To understand the role of hydrogen ions in the control of intracellular ionic environment it is necessary to measure concentrations of other ions such as sodium and potassium. Methods have been developed for observing intracellular sodium by the use of shift reagents (for a review see Springer 1987). We have been investigating the use of relaxation reagents and multiple quantum measurements on ions with a spin of $\frac{3}{2}$ (Payne *et al.* 1990). At the same time we have carried out detailed investigations using rubidium as a replacement for potassium ions and exploiting the

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high sensitivity of the ^{87}Rb NMR signal. We have studied rubidium uptake into erythrocytes (Allis *et al.* 1989*a*), into intact heart (Allis *et al.* 1989*b*), kidney (Endre *et al.* 1989) and into the muscle of live animals (Syme *et al.* 1990*b*). Since over 90% of rubidium is taken up into the cells by the sodium/potassium ATPase, as an analogue of potassium the Rb^+ uptake measurements give us an indication of the NaK/ATPase fluxes *in vivo*. Here I discuss one aspect of our studies using rubidium NMR, that of investigating ionic control in skeletal muscle in essential hypertension (Syme *et al.* 1990*c*).

When we administer a steady dose of 2 mmol kg^{-1} intraperitoneal RbCl to spontaneously hypertensive rats, NMR measurements of rubidium in the muscle show an increased rate of rubidium uptake into the muscle of such animals compared with their appropriate controls. This implies that the NaK/ATPase activity in hypertensive skeletal muscle cells is increased about three fold. During isometric contractions the rate of increase in cytoslic acid was less in hypertensive animals than in controls, and this difference did not result in tetanic muscle stimulation from a change in buffering capacity or lactate production (Syme *et al.* 1990*a*, *b*). We concluded that in essential hypertension the sodium–hydrogen antiport activity is also increased significantly. As a result of these investigations and the suggestion that in the leucocytes in patients with essential hypertension there is also an increase in Na^+–H^+ exchange (Ng *et al.* 1986), we set out to investigate a group of patients with hypertension who at the time of the study were either taken off the drugs or were untreated. Just like in the animal studies, we observed that during graded, aerobic dynamic exercise the intracellular acidification in these patients was less than in controls and the hydrogen ions were cleared more rapidly (Dudley *et al.* 1990). The results suggest a change in proton handling rather than production in the muscle of hypertensive subjects.

5. Phospholipid structures *in vivo*

Far the most prominent peak in the phosphorus spectra of human brain and liver obtained at 2 T is the so-called phosphodiester signal. It has been shown recently that in spectra at higher fields i.e. at 4 T or in animal experiments up to 8 T this peak is considerably decreased. It has also been demonstrated before that some of the mobile phosphodiesters such as GPC and GPE can only account for a relatively small proportion of the observed diester signal (Cerdan *et al.* 1986). Recently we have demonstrated in animal liver and brain slices that the phosphodiester signal has the characteristic features of the signal one obtains from large phospholipid bilayers (Murphy *et al.* 1989).

These experiments were based on observing the field dependence and the response to proton decoupling of the diester signal. We have also studied the field dependence and the response to decoupling of the diester signal from brain white matter and a myelin preparation (P. Kilby, unpublished observations). Our studies of isolated brain tissue and of rat brain *in vivo* led us to conclude that well over 95% of the lipid signal is detected *in vivo*. The major component of this signal is from lipid in the bilayer form. In the brain, particularly deep in the white matter the dominant contribution is from myelin. In the depth resolved rotating frame image this shows up as an increase in the signal as we go deeper into the brain in the adult (Cadoux-Hudson 1990*c*). In infants who have not yet fully myelinated the white matter the diester signal does not increase with depth (Moorcraft *et al.* 1990). In the grey matter,

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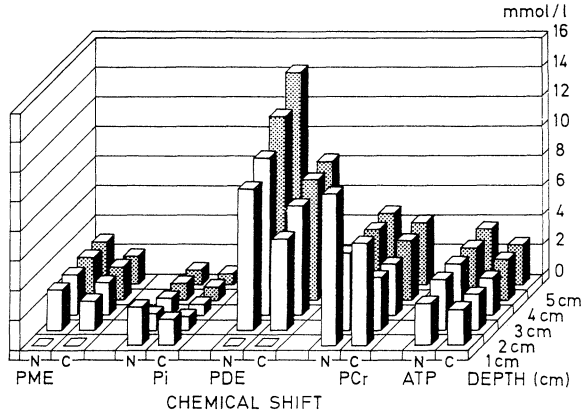

Figure 5. Bar chart comparing mean absolute tissue metabolite concentration from normal subjects (N) and the worst affected hemispheres (contra-lateral to worst spasticity and loss of limb function, (C) of patients recovering from severe head injuries. The darker shading of the bars is used to illustrate depth; darker with depth.

and in human liver, in addition to the lipid bilayer component there is another more slowly relaxing component that is consistent with the signal being derived from a more mobile lipid structure. Thus the diester signal essentially has three components, those from mobile small molecule diesters, those from less mobile but still relatively rapidly tumbling phospholipids and finally the major part of the signal is that from the lipid bilayer which in the brain is dominated by myelin and in the liver is dominated by signals from the endoplasmic reticulum (Murphy 1989).

We have now seen a number of cases of diseased human brain where clear indications of a perturbation in the lipid structure are observed. For example, we have investigated six patients with chronic global head injury and have shown that unlike in the control adult brain the effected side, often many months or years after the initial injury, shows a lack of increase in the diester signal with depth (Cadoux-Hudson 1990*a*) (figure 5). This can be interpreted as resulting from a structural alteration in the deep white matter, presumably replacement of the myelin structures with less dense lipid structures, possibly associated with glyosis. An interesting and unexpected observation associated with this is a change in intracellular pH, a clear acid pH in deep white matter. The reasons for this chronic change are not understood but would be inconsistent with metabolic acidosis and more likely to result from acidosis associated with a new type of perturbed cellular structure such as has been suggested with respect to increased lysosomal contents in some damaged brain cells.

Another area where the study of myelination and demyelination is of importance is in demyelinating diseases and during brain development. In multiple sclerosis we have observed, a substantial lipid signal in the region of the acute plaques in brain but no ATP or phosphocreatine were visible in this region. This observation was made in one patient with acute MS but was absent in the same patient a year later when the plaques initially detected by MRI were also shown to be absent (Cadoux-Hudson *et al.* 1990*a*). We have also studied five other cases with no visible plaques in the brain region where the spectra were normal. We are currently investigating a mouse model for dismyelination, the quaking mouse, where we are beginning to be able to observe lipid changes and structural changes *in vivo*.

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Unfortunately, in the human brain the decoupling experiments that we used in the animal or isolated brain tissue are not feasible for reasons of safety, and so we cannot directly evaluate the phospholipid bilayer signals. There is, however, an alternative way of studying this problem, namely using spin-echo NMR. It is possible to combine the phase-modulated rotating frame imaging experiment with a spin-echo sequence, so that we can directly measure the T_2' relaxation times of the different components in the human brain as a function of depth. Using this method we have been able to show that the major lipid signal is rapidly relaxing and how the values for the T_2' at different depth vary.

6. Conclusions

^{31}P NMR has provided valuable new biochemical information about control, ionic fluxes, enzyme catalysed reactions and phospholipid structures in intact tissues. In particular in over 3000 clinical investigations we have been able to observe some of the underlying biochemical abnormalities in a number of diseases, have been able to elucidate new features of some of the diseases and pinpointed metabolic and structural indices that can be used to evaluate the progression of the disease and its response to various forms of interventions. All these studies require carefully designed biochemical experiments so that the nature of the observation *in vivo* can be interpreted. The studies suffer from limitations in spatial resolution but provided we ask the right questions such resolution does not hinder our ability to derive significant new information about human disease.

The work described results from contributions of many collaborators in the Unit. They are acknowledged through the reference list or in the text as appropriate. The work was supported by the MRC, the Department of Health and the British Heart Foundation.

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