In vivo electron spin resonance spectroscopy: what use is it to gastroenterologists?

N S Dhanjal, I J Cox, S D Taylor-Robinson

Electron spin resonance (ESR) spectroscopy may have a role in the future in assessing the mucosal integrity of the colon non-invasively in the otherwise normal looking colon of patients with quiescent colitis

Like all techniques that strive to bridge the gap between laboratory science and clinical medicine, electron spin resonance (ESR) spectroscopy builds on established applications in biochemistry and chemistry, following on from its discovery by Professor EK Zavoisky and colleagues in 1944 at Kazan State University, situated deep within the Tatarstan Republic of the Russian Federation, formerly the Soviet Union. However, it is only now that developments in technology may perhaps allow the endoscopist of the future to acquire information on gut mucosal integrity in vivo during a procedure. This is an intriguing prospect, although there are a number of practical problems to be solved before the in vivo clinical potential of this sensitive and specific technology is realised. The average endoscopist, faced with the clinical burden of disease and an ever growing case load, requires an emerging clinical technique to robustly deliver reproducible clinically relevant data without obfuscation by artefact. The questions therefore arise of how feasible will it be for ESR spectroscopy to be implemented in the clinical arena and what additional information can be given to the average busy gastroenterologist?

To delve into the basic physics of the technique for a moment, ESR, also known as electron paramagnetic resonance (EPR) spectroscopy, describes the resonant absorption of microwave radiation by paramagnetic materials—that is to say, materials with an unpaired electron such as free radicals and transition metal ions—in the presence of a static magnetic field. Specifically, with respect to in vitro ESR spectroscopy, which is a well used biochemical tool, the sample is placed in a resonant chamber in a magnetic field and microwave frequency is then applied. The resulting ESR spectrum illustrates net absorption of microwaves at a specific frequency, which is dependent on the atomic and molecular structure of the sample under analysis. While an individual electron spin contributes to the magnetic moment of an atom, the majority of materials are not amenable to study by ESR spectroscopy as their electrons are paired and there is therefore no net bulk magnetism. This means that the region under scrutiny must contain a paramagnetic substance and so, for clinical applications, either a free radical must be administered or a so-called ‘spin trap’ must be utilised to provide a mechanism for detection of reactive naturally occurring free radicals, present only in very low concentrations. By way of comparison, nuclear magnetic resonance (NMR) spectroscopy is based on the property of nuclear spin and there are a number of similarities between these two non-invasive techniques. Over the fact that electrons have a greater magnetic moment than nuclei, ESR spectroscopy is more sensitive than NMR spectroscopy. ESR spectroscopy also has the advantage of being highly specific, although it clearly can be a disadvantage that most chemical and biological materials are not paramagnetic. ESR spectroscopy has the scope for studying faster ESR timescale in the time domain is nanoseconds and not milliseconds as in NMR. The ESR technique has more recently been harnessed to study the presence and generation of free radicals in intact cells, perfused organs, and in small animals in vivo. For practical purposes, ESR spectroscopy allows some insight into tissue inflammation through measurement of free radicals. Taken to its logical conclusions in the clinical context, an endoscope with ESR spectroscopy capabilities could, for example, be of use for surveillance when the mucosal surface may otherwise appear normal.

In this issue of Gut, Togashi and colleagues have used ESR spectroscopy to investigate changes in mucosal sulfhydryl compounds in an animal model of colitis [see page 1291]. These authors have previously evaluated the ESR active compound 3-carbamoyl-2,2,5,5-tetramethylpyrrolidine-1-oxyl (carbamoyl-PROXYL) as a ‘spin probe’ for measuring oxidative stress in the murine liver. The same technique has been extended to experimental chronic colitis, it was argued that adequate levels of mucosal sulfhydryl compounds, such as reduced glutathione, are critical in the prevention of tissue damage from the generation of reactive oxygen species in inflammatory conditions, such as ulcerative colitis. This technique provides a non-destructive method of assessing oxidative stress in small animals and these authors have produced a very elegant study using their in house, low frequency, 700 MHz microwave ESR spectroscopy apparatus. The authors are developing new ESR spectroscopy equipment with a surface coil-type resonator, which may be applicable to clinical colonoscopy.

The development of low frequency ESR spectroscopy, combined with the introduction of surface coil resonators, has opened up a wide range of applications for ESR as the depth sensitivity of the technique has improved and the required sample size is less restricted by the dimensions of the resonator. Furthermore, methods of reducing artefacts from voluntary and involuntary motion are being addressed. As with all new techniques, safety issues must be considered as magnetic fields and microwave power are integral to the ESR spectrometer, albeit at low levels, and because paramagnetic materials may be administered. The current generation of ESR spectrometers have quite limited physical space, as illustrated in the equipment used in the study of Togashi and colleagues, and therefore larger magnets are required for interventional clinical applications. With regard to the development time to clinical usage, there are some parallels with NMR spectroscopy. The NMR phenomenon itself was discovered shortly after World War II, but it was not until the mid-1980s that human NMR spectroscopy studies started on liver and in muscle using whole body magnets. In that sense, NMR spectroscopy was ahead of the game compared with ESR spectroscopy but there were still many years of proving the value of NMR spectroscopy before clinical studies were undertaken in earnest. In fact, for gastroenterologists, the liver remains the main focus of interest for NMR spectroscopy as in vivo studies on the gut are fraught with technical difficulties whereas the liver as a solid organ is a much easier focus for NMR study. Therefore, having an endoscope with in built NMR spectroscopy capabilities is still on the drawing board, rather than being a practical reality.

Returning to the problem in hand, the study by Togashi et al. illustrates that it
COMMENTARIES

could be very desirable to have ESR spectroscopy capabilities for a new generation of future endoscopes in order to assess the mucosal integrity of the colon non-invasively in the otherwise normal looking colon of patients with quiescent colitis. However, so that this goal can become a reality, a range of safety and practical issues need to be overcome, obviously initially in the domain of research institutes where clinician scientists can conduct small scale research studies on selected patients with specialist equipment. While there are some potential pitfalls, we do suggest that you follow the development of clinical ESR spectroscopy enthusiastically. Nevertheless, it remains to say that time will tell whether the technique becomes sufficiently robust to join the diagnostic armamentarium of the busy clinical gastroenterologist.

Gut 2003;52:1236–1237

Authors’ affiliations

N S Dhanoj, Department of Medicine A, Division of Medicine, Imperial College London, Hammersmith Hospital Campus, London, UK

I J Cox, Robert Steiner Magnetic Resonance Unit, Imaging Sciences Department, MRC Clinical Sciences Centre, Faculty of Medicine, Imperial College London, Hammersmith Hospital Campus, London, UK

S D Taylor-Robinson, Department of Medicine A, Division of Medicine, and Robert Steiner Magnetic Resonance Unit, Imaging Sciences Department, MRC Clinical Sciences Centre, Faculty of Medicine, Imperial College London, Hammersmith Hospital Campus, London, UK

Correspondence to: Dr S D Taylor-Robinson, Gastroenterology Unit, Imperial College London, Hammersmith Campus, Du Cane Rd, London W12 OHS, UK; s.taylor-robison@imperial.ac.uk

REFERENCES


Cancer

Keeping neuroendocrine cells in check: roles for TGFβ, Smads, and menin?

G J Dockray

Neuroendocrine tumour cells of the gastroenteropancreatic tract are subject to paracrine and autocrine growth inhibition by transforming growth factor β which may account for the low cell proliferation of this tumour

In extreme cases this gives rise to tumours that are called “neuroendocrine” as they exhibit some of the features of neurons and endocrine cells. There are many similarities between neuroendocrine tumours of the gastrointestinal tract and pancreas. In general, these tumours grow slowly and the reasons for this are unknown. Wimmelm and colleagues now present evidence that transforming growth factor β (TGFβ) is produced by neuroendocrine tumours and through autocrine and paracrine mechanisms restrains tumour cell proliferation [see page 1308].

There are over a dozen major enteropancreatic tumour cell (EETC) types, most with a restricted distribution along the gut. The cellular mechanisms that normally determine the differentiation of these cells, and their numbers relative to other epithelial cells in each region of the gut, are only now becoming clear. For example, the basic helix-loop-helix (bHLH) transcription factor neurogenin 3 is required for the development of intestinal and pancreatic endocrine cells and for the main pyloric antral endocrine cells (G and D cells), but not for endocrine cells of the gastric corpus such as enterochromaffin-like (ECL) and X

Neuroendocrine tumour cells of the gastroenteropancreatic tract are subject to paracrine and autocrine growth inhibition by transforming growth factor β which may account for the low cell proliferation of this tumour

The endocrine cells of the gastrointestinal epithelium sense the luminal contents and through secretions at their basolateral side signal both to other epithelial cells and to subepithelial cells, including smooth muscle, neurones, and inflammatory cells. Some of the features of these cells are clearly neurone-like and for a time it was thought that during development they might be derived, like enteric neurones, from the neural crest. This now seems unlikely, and instead it is thought that normally they arise from the pluripotent stem cells that also give rise to the other epithelial cell lineages. However, in some circumstances at least, these cells appear to have the capacity for proliferation, and in

www.gutjnl.com

Downloaded from http://gut.bmj.com/ on October 1, 2017 - Published by group.bmj.com
The extent to which the mechanisms determining EEC differentiation also play a part in the EEC hyperplasias found in different clinical conditions remains uncertain. Clear examples of EEC hyperplasia in patients include ECL cell hyperplasia in hypergastrinaemia, G cell hyperplasia in achlorhydria, and rectal EEC hyperplasia in Campylobacter enteritis. Of these, ECL cell hyperplasia in the stomach is the best understood. Thus hypergastrinaemia in several different clinical settings, including gastrinoma, pernicious anaemia, and chronic atrophic gastritis, is provided by observations in multiple endocrine neoplasia type 1 (MEN-1). Whether this occurs normally in people is uncertain. However, it is observed in both patients with chronic gastritis and experimental animals, hypergastrinaemia is also associated with ECL cell dysplasia and with a tendency to develop ECL cell carcinoid tumours. Moreover, there is evidence that in the setting of pernicious anaemia these tumours may regress after antrectomy compatible with the view that gastrin provides a primary drive to proliferation. At the cellular level, a clue to the mechanisms that might regulate the proliferation of neuroendocrine tumour cells is provided by observations in multiple endocrine neoplasia type 1 (MEN-1). Expression of c-myc in tumours occurs in both MEN-1 and sporadic carcinoid. In addition, loss of heterozygosity (LOH) at the locus of the menin gene occurs in about 75% of ECL cell carcinoid tumours in patients with gastrinoma on a background of MEN-1, compared with <15% of patients with ECL cell carcinoids on a background of hypergastrinaemia due to chronic atrophic gastritis. Interestingly, LOH at this locus was not observed in mid and hindgut carcinoid tumours. These observations implicate the product of the menin gene in the inhibition of proliferation of both pancreatic and gastric endocrine tumours. The relevant protein, menin, binds several signalling proteins, including the transcription factors Jun-D and Smad3. Smad3 is a downstream mediator of TGFβ signalling, and as loss of it appears to downregulate Smad3 function, it seems reasonable to suppose that TGFβ might be a negative regulator of proliferation in at least some neuroendocrine tumours. The idea is attractive not least because TGFβ is known to inhibit the proliferation of other cells.

The data reported by Wimmel et al support the idea that TGFβ inhibits neuroendocrine tumour cell proliferation. The authors showed by immunohistochemistry that TGFβ1 was expressed in 50–80% of fore, mid, and hindgut neuroendocrine tumour cells as well as by mesenchymal cells, and that the two relevant receptors, TGFBR1 and TGFBR2, were also highly expressed by these tumours. There was similar expression in two neuroendocrine cell lines (BON cells, from a functional human pancytokeratin-rich neuroendocrine tumour, and LC1-18 cells from a non-functional colorectal neuroendocrine tumour) and in these cells TGFβ was shown to increase p16INK4A and decrease c-myc, causing arrest in the G1 phase of the cell cycle. Moreover, neutralising antibodies to TGFβ or treatment with a dominant negative receptor, increased proliferation of responsive neuroendocrine cell lines. Taken as a whole, these findings provide direct evidence for the importance of TGFβ as a paracrine/autocrine inhibitor of neuroendocrine tumour cell proliferation. Further experiments are required to determine whether TGFβ is a negative regulator of endocrine cell proliferation. The findings are generally compatible with data in other systems that indicate inhibition of proliferation by TGFβ mediated by the Smad pathway and directed at decreased expression of c-myc and induction of p16INK4A. The role of TGFβ in tumorigenesis is however more complicated. In particular, in other cancers it is now clear that TGFβ can act both as an enhancer of tumour progression as well as a suppressor. The picture emerging over the last few years indicates that TGFβ also stimulates tumour cell migration, promotes epithelial to mesenchymal transition (EMT), and increases the production of matrix metalloproteinases (MMPs); together these effects lead to tumour cell invasion and metastasis. Interestingly, while the Smad signalling pathway appears to be required for inhibition of proliferation, other signalling systems including the MAPK, PI-3, and protein phosphatase2A/p70s6k pathways are implicated in the pro-oncogenic effects of TGFβ. The mechanisms responsible for the shift in TGFβ signalling from a tumour suppressor mode to a tumour enhancer are still unclear. Wimmel et al did not specifically address the question of whether TGFβ stimulates invasion, EMT, or expression of MMPs in neuroendocrine tumour cells. However, as these cells appear to retain the inhibitory effects of TGFβ on proliferation, they may provide a useful model for further studies of the relative importance of the tumour suppressor and pro-oncogenic actions of TGFβ.

Recent reports have suggested possible ways to block TGFβ signalling by delivery of soluble TGFβ receptor protein constructs. 16–18 In experimental models, this approach appears to inhibit tumour cell invasion, and so may be valuable in preventing cancer progression. However, because suppression of neuroendocrine tumour cell proliferation by TGFβ appears to be relatively well preserved, a primary objective in this case should be the maintenance and enhancement of this action of TGFβ. Care should be taken before considering whether inhibition of TGFβ is worthwhile.

Gut 2003;52:1237–1239

REFERENCES
14 Ryberg B, Axelson J, Hakanson R, et al. Trophic effects of continuous infusion of
Inappropriate ileal conservation of bile acids in cholestatic liver disease: homeostasis gone awry

A F Hofmann

Patients with cholestatic liver disease are likely to inappropriately conserve bile acids. Ursodiol corrects the defect, but is this enough?

Conjugated bile acids are water soluble amphipathic end products of cholesterol metabolism that promote lipid transport in the biliary tract and small intestine by forming mixed micelles. Bile acids are formed in pericentral hepatocytes by a complex multi-enzyme process whose details have at last been largely elucidated. After formation, their acidic group is linked ("conjugated") with the amino group of glycine or taurine in an amide bond that is resistant to the proteolytic enzymes present in pancreatic secretion and on the surface of the enterocyte brush border. Conjugated bile acids differ from unconjugated bile acids in being membrane impermeable and water soluble at the pH conditions prevailing in the biliary tract and small intestine.

Efficient ileal conservation of bile acids results in the accumulation of a mass of bile acids termed the bile acid "pool". Between meals, most of the pool is stored in the gall bladder; with meals, the gall bladder discharges bile into the small intestine where bile acids promote lipid absorption. Both bile acid synthesis and ileal conservation continue after a meal but the gall bladder does not increase in volume in proportion to the amount of bile acids it contains because of its continuous concentration of bile. The gall bladder appears early in vertebrate evolution and genes for gall bladder development appear to have evolved at the same time as genes for bile acid synthesis and intestinal conservation.

Development of the enterohepatic circulation and gall bladder storage resulted in far more bile acids being available for digestion than those recently synthesised. Each bile acid molecule is used multiple times before it is lost to the large intestine.

Feedback inhibition of bile acid biosynthesis in the hepatocyte is well established experimentally. Interruption of the enterohepatic circulation causes increased bile acid synthesis. This may be modest, for example, increases of 3–4 times are seen in patients taking bile acid sequestrants for hypercholesterolaemia; or it may be marked, for example, increases of 10–15 times are seen with an ileal resection causing severe bile acid malabsorption. Bile acid feeding of any of the natural bile acids occurring in human bile (cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA)) suppresses bile acid synthesis, but the effect is relatively small (about a 50% decrease).

The mechanism by which the concentration of bile acids in the hepatocyte regulates bile acid synthesis has been elucidated only recently. Bile acids enter the nucleus and bind to a heterodimeric protein composed of two nuclear receptors, FXR and RXR. Binding of the bile acid molecule to FXR changes its confirmation. This in turn leads to a complex sequence of events resulting ultimately in increased synthesis of one or more inhibitory proteins. The inhibitory protein(s) repress(es) the activity of the gene for cholesterol 7 alpha hydroxylase, the rate limiting enzyme in bile acid biosynthesis. FXR, the bile acid nuclear receptor, has now been crystallised, its structure determined by x-ray crystallography, and the shape of the cavity that holds the conjugated bile acid elucidated in the last few months.

Transport of bile acids by the ileal enterocyte is also modulated in a homeostatic manner analogous to feedback inhibition of bile acid biosynthesis in the hepatocyte. Early studies of bile acid secretion at the Mayo Clinic reported that bile acid secretion increased only modestly or not at all when bile acids were fed; hinting at downregulation of bile acid transport in response to bile acid feeding. The first convincing experimental evidence for feedback inhibition of bile acid transport was reported by Lillienau and colleagues who performed experiments in the guinea pig. These workers measured total ileal absorptive capacity for conjugated bile acids by perfusing bile acids at such a high rate that the intraluminal concentration remained constant. This technique had been used previously in studies that defined the Tm for ileal transport in rats and humans. Lillienau et al found that the ileal transport capacity for bile acids decreased after bile acid feeding and was increased by addition of cholestyramine to the diet. This finding was confirmed for the mouse, but using other experimental designs it was not confirmed in the rat (see Lanzini and colleagues) or in the pig. Thus in this area of physiology there are marked species differences, a problem that continues to bedevil those who try to understand the intricacies of bile acid metabolism. The mechanism by which the concentration of bile acids in the ileal enterocyte modulates enterocyte transport is under active investigation at the moment. As in the hepatocyte, regulation is likely to involve interaction of bile acids with nuclear receptors such as FXR.

Lillienau and colleagues speculated that “patients with cholestatic liver disease are likely to inappropriately conserve endogenous dihydroxy bile acids such as CDCA and DCA, which are
known to be hepatotoxic’. This speculation has now been confirmed in an important clinical study by Lanzini and colleagues in this issue of Gut [see page 1371]. These workers used ‘Se- SeHCAT, a selenium tagged homologue of taurocholate, whose metabolism was shown by Jazrawi et al to be essentially identical to that of taurocholate’. Because SeHCAT is a gamma particle emitter, it can be used to visualise the enterohepatic circulation and has been used for this purpose to measure hepatic excretory function non-invasively in patients with cholestatic liver disease. SeHCAT has also been used to measure the efficiency of ileal conservation of bile acids in diarrhoeal conditions.

In the experiments reported by Lanzini et al, SeHCAT was used as a surrogate for taurocholate, and its turnover rate quantified by measuring gall bladder radioactivity daily for several days. The rate of decline in radioactivity with time gives the fractional turnover rate of the endogenous bile acid pool. The method used by Lanzini et al does not provide information on bile acid synthesis, which is the product of pool size and turnover rate.

Lanzini et al found that the fractional turnover rate of 14 women with primary biliary cirrhosis (PBC) was, on average, one half that of 14 age matched healthy women. The TV (equal to 0.69 divided by the fractional turnover rate) was correspondingly increased. Thus in these patients with all stages of PBC, bile acids were inappropriately retained. The simplest interpretation of this novel finding is that the ileum has sensed a lowered intraluminal bile acid concentration and reacted by increasing its efficiency of bile acid conservation. However, a sensing of the elevated plasma level of bile acids might also contribute. In health, the ileum efficiently downregulates transport in response to increased bile acid loads thereby protecting the liver. When the bile acid pool is lost, as in acute diarrhoeal disease, the ileum upregulates to regenerate the bile acid pool as quickly as possible. In cholestatic liver disease, the signal of decreased intraluminal bile acid concentration acts to mislead the ileal transport system, which cannot know that bile acids are being retained in the hepatocyte because of biliary ductule obstruction. Inappropriate ileal conservation in cholestatic liver disease is homeostasis gone awry.

Lanzini et al made a second important observation. Inappropriate ileal conservation of bile acids was abolished by administration of ursodiol at the usual dose of 15 mg/kg/day. Although ursodiol is fairly well absorbed, it does not suppress endogenous bile acid synthesis because it does not interact with the nuclear receptor FXR. Thus in patients receiving ursodiol, the enterohepatic circulation has an additional input (probably 10–12 mg/kg/day) of exogenous bile acids, far exceeding endogenous bile acid synthesis (3–5 mg/kg/day). Presumably, ursodiol conjugates secreted by the liver compete for active ileal transport, thus preventing the inappropriate conservation of endogenous bile acids and restoring the fractional turnover rate to normal. Ursodiol is non-cytotoxic and has multiple effects on the hepatocyte that appear to decrease the injurious effects of retained endogenous bile acids and to promote hepatic excretory function.

A major question remaining for the hepatologist is whether downregulation of ileal bile acid transport to its normal level by ursodiol therapy is optimal therapy in cholestatic liver disease, or whether it is desirable to further increase the efficiency of ileal conservation to a still greater degree, thereby reducing the return of bile acids to the hepatocyte that is already impacted with bile acids.

Historically, bile acid drainage was used to treat the pruritus of cholestatic liver disease. When cholestyramine was introduced, it was also shown to decrease pruritus, and that, and still now, is considered by many to arise from increased plasma levels of bile acids. Emerick and Whittington have treated intractable pruritus in children by partial biliary diversion which prevents a fraction of secreted bile acids from reaching the ileum. Another surgical approach reported to be successful is ileal bypass which should have the same effect as partial biliary diversion. The technique of extracorporeal albumin dialysis removes plasma bile acids and decreases pruritus. A new bile acid sequestrant, colesveal, has binding properties for bile acids that are superior to those of cholestyramine and has been reported to be more effective than cholestyramine in treating cholestatic pruritus in open label studies. The majority of these cholestatic patients were already receiving ursodiol so that these adjuvant therapeutic approaches appear to add efficacy to that achievable by ursodiol therapy alone. All of these approaches will result in less absorption of endogenous cytotoxic bile acids so that the input of bile acids to the liver will be enriched in the recently ingested ursodiol.

The last approach to be considered is inhibition of asbt, the apical transporter of the ileal enterocyte. Ileal absorption of bile acids begins with transport into the enterocyte mediated by the apical sodium dependent bile acid transporter (asbt) that has been cloned and characterised in the laboratory of Dawson. Development of a potent inhibitor of asbt has been the goal of several pharmaceutical companies. The target disease for such an inhibitor of bile acid transport was not cholestatic liver disease, but hypercholesterolaemia, a far more prevalent problem. The rationale for the development of such inhibitors was the observation that addition of a bile acid sequestrant to a statin potentiates its hypcholesterolaemic effect by still further upregulating LDL receptor activity. Sequestrants are known to induce only mild bile acid malabsorption, suggesting that a potent asbt inhibitor (together with a statin) should be still more effective therapy for hypercholesterolaemia. Although statins do not protect against the development of gallstones, it is to be hoped that, when combined with appropriate bile acid transporters, these agents might reduce the risk of gallstone development in patients with gallbladder disease. Incompetent ileal transporters appear to be less of a problem in cholestatic patients as the compensatory increase in bile acid synthesis might be dampened because of liver disease.

The paper of Lanzini et al is an important advance in our understanding of the pathophysiology of cholestatic liver disease. The enterohepatic circulation of bile acids arose in vertebrate evolution to promote nutrition, not to deal with the problem of cholestatic liver disease. Ursodiol therapy corrects the defect in inappropriate conservation. Whether this is enough or whether we should further reduce ileal transport can be tested if the newly developed asbt inhibitors become available to the liver community. Still, all of the approaches discussed above are palliative and we must continue to seek therapeutic approaches that deal with the fundamental aetiology of these conditions, which is likely to be infectious and/or autoimmune.

Author’s affiliation
A F Hofmann, Division of Gastroenterology, Department of Medicine, University of California, San Diego 92093–0813, USA; afhofmann@ucsd.edu

REFERENCES


34 Vega GL, Grundy SM. Treatment of primary moderate hypercholesterolemia with lovastatin (mevastatin) and colestipol. JAMA 1987;258:33–8.
Inappropriate ileal conservation of bile acids in cholestatic liver disease: homeostasis gone awry
A F Hofmann

Gut 2003 52: 1239-1241
doi: 10.1136/gut.52.9.1239

Updated information and services can be found at:
http://gut.bmj.com/content/52/9/1239

These include:

References
This article cites 29 articles, 5 of which you can access for free at:
http://gut.bmj.com/content/52/9/1239#BIBL

Email alerting service
Receive free email alerts when new articles cite this article. Sign up in the box at the top right corner of the online article.

Notes

To request permissions go to:
http://group.bmj.com/group/rights-licensing/permissions

To order reprints go to:
http://journals.bmj.com/cgi/reprintform

To subscribe to BMJ go to:
http://group.bmj.com/subscribe/