

Haematopoietic cancer

## Clinical epidemiology—how important now?

V Binder

### The putative risk of haematopoietic cancer in relation to modern strong immunosuppressive treatment in inflammatory bowel disease

Chronic diseases have become an increasing portion of modern medicine in the Western world and the goals for treatment are not only to relieve symptoms and their influence on daily life for patients but also to avoid long term complications of the disease and of the treatment given.

The rules for obtaining evidence of the benefit of a new treatment have been widely accepted, with the randomised controlled study as the “gold standard”. Such studies give some guarantee of the effect of a given treatment modality. The possible long term side effects however cannot be secured against in studies carried out over a few months, the usual length of a controlled trial.

The natural history of a chronic disease—how will the disease proceed if no medical intervention is carried out—should ideally be the background for any therapeutic trial. For the immediate short term course, the placebo arm of a controlled study is sufficient. Knowledge of the long term natural course of the disease however does not exist as both the medical profession and patients themselves have continuously interfered and tried to relieve the consequences of the disease. Gradually therefore, the meaning of “natural course” has changed to “the course of the disease when treated in accordance with established and well defined treatment policies”. Studies of this kind, systematic collection of clinical data, have extended the subject of clinical epidemiology from estimation of incidence and prevalence of diseases in a population, to long term follow up of cohorts of patients. Good epidemiological studies imply avoidance of selection of patients using regional patient cohorts, securing complete follow up with a minimum of untraced patients, and use of well described and unambiguous definitions of diagnosis and clinical parameters. If the pitfall of selection is not avoided, the created patient group may be unrecognisable, and the results obtained become misleading.

Modern information technology has facilitated the creation of large databases which, combined with epidemiologically correct data collection, give the possibility of depicting prognosis and the long term course of chronic diseases during a given treatment regimen. Although it is both costly and time consuming to maintain such databases, it is our professional responsibility to cumulate clinical experience in order to inform patients properly and to find strategies for improvements in treatment.

With regard to inflammatory bowel disease, the above mentioned goals for medical management of these chronic diseases are mandatory. The development of new biological treatment modalities, such as infliximab, the tumour necrosis factor  $\alpha$  inhibitor with strong immunosuppressive properties and a well documented effect on Crohn's disease, has been the major achievement within the last half decade. The long term effect however, and in particular the fear of increased risk of lymphoma, has been a matter of concern. In this issue of *Gut*, a Swedish group<sup>1</sup> of very experienced clinical epidemiologists have used their two regional population based cohorts of patients with ulcerative colitis and Crohn's disease—more than 8000 patients—diagnosed between 1955 and 1990, to examine the occurrence of haematopoietic disease during the course of IBD (see page 617). Furthermore, they added a nationwide inpatient register of 45 000 patients diagnosed between 1964 and 2000. All patients were identified by an individual national registration number and linked with the Swedish cancer register. Patients from the regional cohorts and national inpatient register were analysed separately, but the results were very similar—namely, a non-significant, borderline increased risk of lymphoma in patients with Crohn's disease and a borderline increased risk of myeloid leukaemia in ulcerative colitis. Interestingly, the lymphoma risk appeared within the first five years of Crohn's disease. Most of the observation

period was before the era of infliximab treatment and immunosuppression with azathioprine but a limitation of the study was the lack of information about pharmacotherapy in the patients. None the less, the study is important and calls for close follow up of future patients with IBD, especially those receiving new biological treatment modalities. Such a study, also from Sweden, was published very recently by Ljung and colleagues.<sup>2</sup> IBD patients from Stockholm county were all treated with infliximab within a two year period and severe adverse events were noted in 19% of patients and a 1.5% annual incidence of lymphoma. This study however was hampered by not having any controls.

The tool for monitoring new treatment modalities in relation to already established management of patients will be ongoing previous and future studies based on lifelong close follow up of patients in outpatient clinics. Such studies reveal a detailed picture of the disease course in each patient and in subgroups of patients, classified according to clinical characteristics and treatment. There is of course much more that characterises and influences the long term course of inflammatory bowel disease than just the complication lymphoma. Studies from Denmark in ulcerative colitis, carried out over 3–4 decades,<sup>3–4</sup> have revealed survival not different from that of the background population from one year after diagnosis to 36 years. Further analysis showed that the subgroup of patients with extensive colitis and late onset of disease (>50 years), who demanded surgical intervention within the first year of their disease, were at a higher risk of dying from postoperative complications or comorbidity. Leukaemia and lymphoma were not found more frequently than expected in the population in general. This cohort of patients was treated in the pre-immunosuppressive era and had only short courses of corticosteroids at flare ups.

In Crohn's disease, slightly increased mortality was observed in women with Crohn's disease late in the disease course, 20–25 years after diagnosis, associated with severe inflammatory bowel disease.<sup>5–6</sup> No lymphomas were found, and in general the risk of extraintestinal cancer was not different from those without Crohn's disease. Cohort studies with continuous clinical follow up over long periods of time have been published in Sweden,<sup>7–8</sup> Italy,<sup>9</sup> and the USA,<sup>10</sup> all necessary background for further studies which need to be carried out as more aggressive and more effective medical treatment modalities become more common. There is no evidence that inflammatory bowel disease per se carries an increased risk of lymphoma.

Electronic patient records will apparently be generally available within the coming years, at least for outpatients, but soon for all patient care, in order to secure the quality of treatment. It must be borne in mind that information technology is only a tool, especially for administrators, and it can never replace the knowledge and expertise of clinical epidemiology and good clinical practice. It is our responsibility that these skills are implemented in the IT future of medicine.

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Correspondence to: Dr V Binder, Herlev Hospital, DK 2730 Herlev, Copenhagen, Denmark; vibeke@binders.dk

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## Gall stones

# Increased deoxycholic acid absorption and gall stones in acromegalic patients treated with octreotide: more evidence for a connection between slow transit constipation and gall stones

A F Hofmann

Acromegalic patients treated with octreotide have prolonged colonic transit, increased bacterial formation, and subsequent absorption of deoxycholic acid that is indicated by an increased proportion of deoxycholic acid in plasma bile acids. Enrichment of deoxycholic acid in the circulating bile acid pool leads to supersaturated bile and cholesterol gall stones.

Thomas and colleagues,<sup>1</sup> in this issue of *Gut*, extend previous work from the laboratory of R Hermon Dowling by showing that acromegalic patients treated with octreotide have an increase in faecal anaerobic bacteria that convert cholic acid, the major primary bile acid in humans, to deoxycholic acid, the major secondary bile acid (see page 630). The paper also confirms previous reports from the Dowling laboratory<sup>2,3</sup> that octreotide treatment increases colonic transit time in acromegalic patients. In addition, the paper of Thomas *et al* shows that octreotide treatment results in enrichment of deoxycholic acid in fasting state plasma bile acids.

The present paper is the 11th and could well be the last in a series of

papers spanning more than a decade from the Dowling laboratory that have dealt with the pathogenesis of gall stones in acromegalic patients treated with octreotide, a potent somatostatin agonist. Octreotide is a synthetic peptide whose amide bonds are resistant to hydrolysis by plasma peptidases, thus resulting in its having a much longer duration of action than that of somatostatin.<sup>4</sup> In acromegalic patients, octreotide administration suppresses the release of growth hormone and insulin growth factor by the pituitary and results in clinical improvement.<sup>5</sup>

The initial paper in this series showed that such patients have an increased prevalence of gall stones.<sup>6</sup> The gall stones were shown to be rich in cholesterol<sup>7</sup> and, as would be expected, bile

was found to be supersaturated in cholesterol<sup>8</sup> and to dissolve when patients received ursodiol orally,<sup>7</sup> an agent that is well known to decrease the cholesterol saturation of bile and induce gradual dissolution of cholesterol gall stones.<sup>9</sup>

In addition, previous papers have shown that such patients have impaired gall bladder contraction<sup>2</sup> and prolonged small intestinal and colonic transit,<sup>2,3</sup> as well as an increased proportion of deoxycholic acid in biliary bile acids.<sup>8</sup> To define a mechanism for the increased biliary deoxycholic acid, methods for assessing bacterial deconjugating and dehydroxylating activity in intestinal or faecal content were developed,<sup>10</sup> as these enzymatic steps are necessary for the conversion of cholic acid to deoxycholic acid. Such methods were used in the present paper to show increased dehydroxylating activity (per gram of faecal protein or wet weight). A previous study<sup>11</sup> showed that colonic absorption of newly formed deoxycholic acid was increased in acromegalic patients treated with octreotide. This excellent study used the isotope dilution method for characterisation of bile acid kinetics developed a half century ago by Lindstedt (reviewed by Hofmann and Hoffman<sup>12</sup>). Moreover, there was a linear correlation between colonic transit (presumably the independent variable) and deoxycholic acid input.<sup>11</sup>

The increased colonic absorption was explained by two factors. Firstly, the residence time of colonic content was prolonged. Secondly, it was proposed, based on previous studies of others,<sup>13</sup> that slow colonic transit leads to more alkaline colonic content (although this was not actually measured) and that if luminal contents of the colon were more alkaline, more deoxycholic acid should be in solution. If more deoxycholic acid

is in solution, more should be absorbed, as deoxycholic acid is highly membrane permeable. To prove that the solubility of deoxycholic acid increases as pH is raised, *in vitro* studies of deoxycholic acid solubility in caecal content were performed. These studies showed that the solubility of deoxycholic acid increased linearly with increasing pH.<sup>14</sup> However, binding of deoxycholic acid to luminal contents appeared to influence the pH solubility relationship, because theory predicts that solubility should increase exponentially rather than linearly with increasing pH—that is, in theory, for each unit increase in pH, solubility should increase 10-fold.<sup>15</sup> Finally, studies on gall bladder bile samples showed that the greater the deoxycholic acid proportion in biliary bile acids, the greater the cholesterol saturation and the shorter the crystal detection time.<sup>16</sup> It was shown that bile that was enriched in deoxycholy conjugates had vesicles containing a higher cholesterol/phospholipid ratio, presumably because dihydroxy bile acids have a lower critical micellisation concentration than trihydroxy bile acids. Therefore, for a given bile acid concentration, dihydroxy bile acids solubilise more phospholipid in mixed micelles than trihydroxy bile acids. Such phospholipid solubilisation depletes the phospholipid from phospholipid-cholesterol vesicles in bile, leaving cholesterol rich vesicles that nucleate cholesterol more rapidly.<sup>17</sup>

One must express admiration for the tenacity with which this multifaceted problem has been pursued as well as for the great variety of experimental approaches that have been used. Certainly new insights into deoxycholic acid metabolism have been developed. In addition, a hypothesis for the pathogenesis of the gall bladder stones occurring in acromegalic patients receiving octreotide has been advanced, and a number of lines of evidence supporting this reasoning have been advanced. The purpose of this brief commentary is to highlight the achievements to date and point out a few remaining experimental approaches that seem to be needed to clinch the argument.

The first question is whether octreotide administration *per se* induces the formation of supersaturated bile in the acromegalic patient. Supersaturated bile is not uncommon in the healthy Caucasian adult<sup>18</sup> and appears to be frequent in acromegalic patients, at least in one study.<sup>19</sup> Growth hormone administration to healthy subjects<sup>20</sup> or to children deficient in growth hormone<sup>21</sup> does not alter biliary lipid composition. In a small prospective study, Erlinger *et al* found that acromegalic patients had bile that was supersaturated in

cholesterol and that octreotide administration did not increase the degree of cholesterol supersaturation but was associated with the appearance of cholesterol crystals.<sup>19</sup> These results differ from those of the Dowling group who found that octreotide administration caused biliary cholesterol saturation to increase from unsaturated to supersaturated.<sup>8</sup> Thus it seems that most acromegalic patients who are treated with octreotide begin with, or because of octreotide therapy develop, gall bladder bile that is supersaturated in cholesterol and will rapidly form cholesterol crystals.

In addition to its effect on biliary lipid composition, octreotide administration causes a profound disturbance of biliary tract motility. Octreotide abolishes both cholecystokinin release from the small intestine<sup>22</sup> as well as the contractile response of the gall bladder to infused cholecystokinin.<sup>23</sup> Therefore, at first glance, the effect of octreotide should be to cause a “non-functioning” gall bladder. However, an additional effect of octreotide should be to inhibit the usual prandial relaxation of the sphincter of Oddi, an action now known to be mediated by local release of nitric oxide.<sup>24</sup> The coordinated contraction of the gall bladder and relaxation of the sphincter of Oddi leads to controlled emptying of gall bladder contents into the duodenum during digestion, which is evidenced by the postprandial rise in the plasma bile acid level.<sup>25</sup> Remarkably, the flow and ebb of the bile acid pool stimulated by meal ingestion is perturbed relatively little by cholecystectomy, as in cholecystectomised patients the pool is stored in the small intestine rather than in the gall bladder, and quickly transported to the distal ileum and absorbed when a meal is ingested.<sup>25</sup>

Bile acid secretion can be assessed indirectly by changes in the plasma bile acid level as fractional hepatic extraction of bile acids remains constant irrespective of the load.<sup>26</sup> At present, we have no information on the dynamics of bile acid secretion in the acromegalic patient treated with octreotide. However, it is reasonable to propose that bile acid secretion is markedly impaired in acromegalic patients because of the paralysis of the gall bladder and sphincter of Oddi. An intraluminal bile acid deficiency is likely to contribute to the steatorrhoea that has been observed in patients with somatostatinomas<sup>27</sup> as well as in patients with other maladies treated with octreotide.<sup>28</sup> Patients with the AIRE syndrome may also have impaired cholecystokinin release and have been shown to have an intraluminal bile acid deficiency.<sup>29</sup> Indeed, malabsorption of

fatty acids as such has been shown to result from octreotide administration,<sup>30</sup> an effect that is best explained by a decreased intraluminal bile acid concentration.

If bile acid secretion is impaired in acromegalic patients treated with octreotide, this could provide an explanation for the formation of supersaturated bile. Older clinical studies showed that during overnight fasting, bile acid secretion decreased to a greater extent than cholesterol secretion, increasing the saturation of bile.<sup>31</sup> The one problem with this line of reasoning is that when bile is stored for prolonged periods in the gall bladder, cholesterol is absorbed to a greater extent than phospholipid or cholesterol, decreasing the saturation of bile.<sup>32–33</sup> The gall bladder bile samples obtained by the Dowling group were obtained by percutaneous puncture, and we have no idea how long the bile had been present in the gall bladder. It would have been possible to determine such by using a biliary recovery marker such as indocyanine green,<sup>34</sup> but this was not done.

Does octreotide have a direct effect on hepatocyte secretion of cholesterol? Biliary cholesterol secretion is still poorly understood although the two ATP stimulated efflux pumps that mediate biliary cholesterol secretion into canalicular bile, *ABC5* and *ABC8*, have recently been identified.<sup>35</sup> The effect of somatostatin on bile flow has received considerable attention<sup>36</sup> but the effect of this hormone or its agonists on biliary cholesterol secretion has had relatively little attention. None the less, Marteau *et al* noted that when somatostatin was administered to a patient with a biliary fistula, bile became supersaturated in cholesterol.<sup>37</sup>

Another question is why gall bladder atony and its colonic equivalent—prolonged colonic transit—develop when the acromegalic patient is treated with octreotide. As noted above, failure of gall bladder contraction during meals is easily explained by the dual action of octreotide. Constipation is presumed to result from a direct inhibitory effect of somatostatin on colonic propulsive motility.<sup>38</sup>

The extensive studies of the Dowling group have shown elegantly that prolonged intestinal transit leads to increased deoxycholic acid absorption from the colon. The paper by Thomas *et al* in this issue of *Gut*<sup>1</sup> shows that faecal samples contain increased dehydroxylating activity in faecal samples. In a previous study, she and her coworkers showed that bacterial activity of faecal samples is similar to that of caecal samples.<sup>39</sup> None the less, as bacterial density is usually expressed in logarithmic units, the

increased bacterial density reported by Thomas *et al*, although statistically significant, is really quite modest.

The Dowling group has argued strongly for a key role of deoxycholic acid in the pathogenesis of supersaturated bile and cholesterol gall stones in these patients based on their observation that increased biliary deoxycholic acid was associated with increased biliary cholesterol saturation and rapid cholesterol crystal formation *in vitro*. Older studies of Marcus and Heaton show clearly that slowing colonic transit increases biliary deoxycholic acid and increases biliary cholesterol saturation.<sup>40</sup> The Carulli group showed that enriching bile in deoxycholic acid by feeding cholic acid, its precursor, induced the formation of supersaturated bile.<sup>41</sup> Berr *et al* identified a group of gall stone patients with greatly increased deoxycholic acid formation. When these patients were given ampicillin to decrease deoxycholic acid formation, bile became less saturated in cholesterol.<sup>42</sup> Additional mechanisms by which increased deoxycholic acid absorption can promote the formation of cholesterol gall stones are summarised in a scholarly review by Van Erpecum and Van Berge-Henegouwen.<sup>43</sup> A novel hypothesis by which increased deoxycholic acid input could cause increased biliary cholesterol saturation was first advanced by Einarsson *et al*<sup>44</sup> based on studies in healthy subjects. These workers found that deoxycholic acid feeding causes inhibition of cholesterol 7 $\alpha$ -hydroxylase activity (presumably by activating FXR, the nuclear receptor that modulates bile acid synthesis). At the same time, deoxycholic acid did not inhibit HMG CoA reductase activity (presumably by not activating the PPAR $\alpha$  nuclear receptor<sup>45</sup>). This disassociation of cholesterol synthesis from cholesterol catabolism into bile acids provides a possible mechanism for the induction of supersaturated bile secretion by deoxycholic acid.

None the less, the two largest studies of biliary bile acid composition and biliary cholesterol saturation<sup>46–47</sup> have found little relationship between the proportion of deoxycholic acid and the extent of supersaturation with cholesterol. Moreover, when deoxycholic acid was given to healthy volunteers at rates exceeding the usual input by a factor of 7–10, little change in biliary cholesterol saturation occurred.<sup>48</sup>

We are left with the intriguing possibility, also proposed by others, that deoxycholic acid may have a biphasic dose-response curve: low doses (or input) in the range of the normal daily input from the colon increase biliary cholesterol saturation whereas higher

(pharmacological) doses do not alter biliary cholesterol saturation. Can this be tested? What is needed is a simple dose-response study of oral deoxycholic acid, as has been performed for chenodeoxycholic acid and ursodeoxycholic acid.<sup>49</sup> This should be done first in acromegalic patients receiving octreotide, to establish the veracity of the hypothesis developed by the Dowling group, and second, in cholesterol gall stone patients.

A final question is whether the formation of deoxycholic acid matters for any other reason. Bile acids are bacteriostatic and their high aqueous concentration in the small intestine contributes to its relative sterility.<sup>50</sup> In the colon, bacterial deconjugation and dehydroxylation converts conjugates of cholic acid to deoxycholic acid (and chenodeoxycholic acid to lithocholic acid); these secondary bile acids are poorly soluble at caecal pH, permitting bacteria to flourish. For herbivores, bacteria mediate the conversion of unabsorbed carbohydrate to short chain fatty acids, which are an important and even essential caloric source for hind gut fermenters such as the horse. Thus, in herbivores, formation of deoxycholic acid which results in a profound decrease in the aqueous concentration of luminal bile acids appears to be beneficial to the whole organism. Humans, however, obtain few calories from their colon,<sup>51</sup> and probably most adults wish that they absorbed none.

Experimental oncologists however take a dim view of deoxycholic acid. A recent study by Pai *et al* has claimed that low concentrations of deoxycholic acid increase tyrosine phosphorylation of beta catenin and enhance colon cancer cell proliferation and invasiveness.<sup>52</sup> Deoxycholic acid has also been reported to induce COX-2 activity<sup>53</sup> and to activate epidermal growth factor receptor.<sup>54</sup> A cautionary note seems in order as these cancer promoter activities of deoxycholic acid have been shown in colon cell lines. Such an experimental situation may not mimic the *in vivo* situation because bile acids may not accumulate in the colonic epithelium if the blood supply is ample. Bovids presumably have a continuing flux of deoxycholic acid across their colonic epithelium, and to the best of my knowledge colon cancer is rare in bovinds.

The extensive body of work from the Dowling group has greatly enriched our knowledge of the factors influencing the absorption of deoxycholic acid from the colon, a silent process that is occurring in all of us. The acromegalic patient treated with somatostatin has multiple “lithogenic” defects in his or her biliary

tract—bile that is supersaturated with cholesterol, a short nucleation time, and impaired gall bladder emptying that provides time for cholesterol crystals to form and sink to the bottom of the gall bladder. In addition, the sphincter of Oddi is unlikely to relax and as a consequence bile acid secretion is likely to be grossly deficient, an additional factor promoting the secretion of bile that is supersaturated in cholesterol. As discussed above, a number of lines of evidence suggest that increased absorption of deoxycholic acid from the colon promotes the secretion of bile that is supersaturated in cholesterol. None the less, deoxycholic acid input needs to be varied experimentally in order to clinch the view that increased deoxycholic acid is a major contributor to gall stone formation in both acromegalic patients receiving octreotide as well as in patients who have idiopathic cholesterol gall stone disease.

Deoxycholic acid and ammonia appear to be the two toxins that we absorb continuously from our colon and that cause little impairment of health so long as the liver is detoxifying normally and the gall bladder is contracting nicely. Based on older work of Heaton<sup>55</sup> and the recent studies of the Dowling group, we now have a hypothesis supported by a great deal of experimental and clinical evidence that can explain the association between constipation caused by slow colonic transit and cholesterol gall stones.<sup>56</sup> Development of this principle is a major achievement in gastroenterology in my judgment.

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Correspondence to: Professor A F Hofmann, Department of Medicine, MC 0813, University of California, San Diego, La Jolla CA 92093-0813; ahofmann@ucsd.edu

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Colorectal cancer

## Reversal of DNA hypomethylation by folic acid supplements: possible role in colorectal cancer prevention

J C Mathers

### Low folate intake may predispose to a greater risk of colorectal cancer

The deranged gene expression that is causal for colorectal cancer (CRC) development results from un-repaired genomic damage which accumulates in successive generations of mucosal cells and which provides the neoplasm with a growth and/or survival advantage. Such damage includes disabling mutations in tumour suppressor (TS) genes and facilitates inappropriate expression of oncogenes. Approximately 25 years ago, evidence began to appear that changes in methylation status of DNA might be responsible for changes in gene expression. The potential importance of aberrant DNA methylation in tumorigenesis was signalled when, on 6 January 1983, *Nature* carried an article by Feinberg and Vogelstein<sup>1</sup> reporting the first evidence of hypomethylation of some genes in tumours compared with "normal" tissue from the same individuals. That same year, Gama-Sosa and colleagues<sup>2</sup> used high performance liquid chromatography to demonstrate that metastatic tumours had a significantly lower content of 5-methylcytosine than did benign neoplasms or normal tissues. Somewhat surprisingly, site specific hypermethylation of particular genes, including the classical TS the retinoblastoma gene, was observed a few years later (see Feinberg and Tycko<sup>3</sup> for an excellent review). It is now clear that global genomic hypomethylation and hypermethylation of specific genes coexist in the same tumours but whether the genesis of the two phenomena is related remains uncertain.

#### ROLE OF ABERRANT DNA METHYLATION IN CANCER DEVELOPMENT

Laird<sup>4</sup> has argued that cancer may be as much a disease of misdirected epigenetics as it is of genetic mutations. Epigenetics describes non-coding changes to the genome which are transmitted through mitosis and alter gene expression. Although changes in other epigenetic markings, especially in post translational modifications of

histones, are likely to be of considerable significance in tumour pathogenesis, much of the work to date has focussed on DNA methylation. Approximately 1% of DNA bases in the human genome is accounted for by 5-methylcytosine which occurs predominantly in CpG dinucleotides. The promoter regions of about half of human genes contain CpG islands which are contiguous windows of 500 or more base pairs in which the G:C content is at least 55% and the observed over expected CpG frequency is at least 0.65.<sup>4</sup> During cell division, methylation patterns in the parental strand of DNA are maintained in the daughter strand by the action of DNA methyltransferase 1 (DNMT1) which catalyses the transfer of a methyl group from S-adenosyl methionine (SAM; the universal methyl donor) to the 5' position on cytosine residues by a relatively complex mechanism.<sup>5</sup>

Unlike cytosines elsewhere in the genome, those in CpG islands are normally unmethylated. Methylation of these CpG islands is accompanied by the transcriptional silencing of a range of genes important in tumorigenesis, including the colorectal mucosal gatekeeper *APC*, the cyclin dependent kinase inhibitor encoded by *CDKN2A*, and DNA repair genes most notably *MLH1* and *MGMT* although whether hypermethylation is causal in this process continues to be debated.<sup>5,6</sup> Given the TS roles of the protein products of these genes, the aetiological consequences of promoter hypermethylation for tumour development are likely to be indistinguishable from the effects of transcriptional silencing by mutation. Not all genes with CpG islands in their promoters become hypermethylated in cancers. For example, *MLH1* and *MSH2* are both DNA mismatch repair genes with CpG islands but only *MLH1* is hypermethylated (observed in up to one fifth of bowel cancers). This suggests that, for reasons unknown, some genes are differentially sensitive to methylation.<sup>6</sup>

Early studies of DNA hypomethylation in tumorigenesis suggested a role in activation of oncogenes such as *HRAS* and *KRAS*<sup>7</sup> which has been confirmed for several additional genes.<sup>3</sup> Knockout of the housekeeping DNA methyltransferase *Dnmt1* in mouse somatic cells resulted in widespread gene activation.<sup>8</sup> However, oncogenic activation is unlikely to be the only means by which global hypomethylation contributes to neoplasia. Hypomethylation appears to induce genomic instability through abnormalities in chromosomal segregation processes<sup>9</sup> and elevated mutation rates.<sup>10</sup> However, neither point mutations nor genomic rearrangements were increased in a separate study of *Dnmt1* deficient mouse embryonic stem cells.<sup>11</sup>

#### FOLATE AND CRC RISK

Diet, lifestyle, and other non-genetic factors have a strong influence on CRC risk, with obesity and possibly red or processed meat increasing the risk while higher intakes of plant foods may be protective.<sup>12</sup> Several components of plant foods may inhibit, reverse, or retard tumorigenesis, with intracellular signalling cascades as the common molecular targets.<sup>13</sup> Higher intakes of the B vitamin folate or higher folate status are associated with a lower risk of bowel cancer (see Kim<sup>14</sup> for review). Plausible mechanistic arguments can be advanced for this observation since (i) folate in the form of 5'-methyltetrahydrofolate is required for the synthesis of SAM and, therefore, for DNA methylation and (ii) 5', 10'-methylene tetrahydrofolate donates a methyl group to uracil converting it to thymine for DNA synthesis. When folate status is low and thymine supplies are limited, dUTP is incorporated into DNA in place of dTTP during DNA replication and repair. The resulting U:A mismatches initiate a futile cycle of DNA repair with single and double strand breaks and chromosomal damage as probable sequelae.<sup>15</sup>

To date, at least seven relatively small (1–20 subjects) human intervention trials of the effects of folate supplementation on putative biomarkers of CRC risk measured in the colorectal mucosa have been published (see Pufulete and colleagues<sup>16</sup> for details of some) but all of these have used relatively large doses of folic acid (1–15 mg/day). In the randomised, double blind, placebo controlled intervention study reported by Pufulete *et al* in this issue of *Gut*,<sup>16</sup> global genomic DNA methylation and folate status were assessed in patients with adenomatous polyps before and after supplementation with 400 µg folic acid/day (twice the reference nutrient intake for this vitamin) or placebo for 10 weeks (see page 648). As expected, circulating

concentrations of folate increased and homocysteine concentrations fell following folate supplementation and there was a 31% increase ( $p < 0.001$ ) in global DNA methylation in leucocytes. Earlier studies by others have shown that a few weeks of moderate folate depletion resulted in global hypomethylation of circulating white cells but the effects on DNA methylation of repletion with folate for similar time periods were inconsistent.<sup>17–19</sup> However, the usefulness of leucocyte DNA methylation as a surrogate for that in other tissues and especially for the colonic mucosa is not known. Pufulete and colleagues<sup>16</sup> have made the novel observation that supplementation with physiologically normal amounts of folate increased genomic DNA methylation in biopsies of colonic mucosa by 25% (albeit, not significantly,  $p = 0.09$ ).

### UTILITY OF ABNORMAL GLOBAL DNA METHYLATION AS A BIOMARKER OF CRC RISK

Having observed a similar degree of hypomethylation of DNA in benign polyps as in malignant tissue, Goelz and colleagues<sup>20</sup> proposed that methylation changes precede malignancy and “could be a key event in the initiation of malignancy”. While it is probable that changes in global methylation which result in activation of oncogenes or genomic instability contribute to the development of neoplasia, it is not clear that all changes in genomic DNA methylation will have such an effect. Much of what is known about the functional effects of altering global DNA methylation comes from studies in which very large changes have been induced by treatment of cells with demethylating drugs (for example, 5-azacytidine)<sup>9</sup> or by knockout of Dnmt1.<sup>8,10</sup> Because of their potential importance in advancing understanding of the mechanistic basis of dietary protection against CRC, the functional consequences of the more subtle effects produced by alterations in nutrient (for example, folate) supply<sup>16</sup> is now a priority for further research. Such research should include gene expression studies employing transcriptomics and/or proteomics approaches to reveal global changes in expression which may accompany the global changes in methylation.

The most widely used methods for assessing global DNA methylation (including the *in vitro* acceptance assay<sup>21</sup> and the cytosine extension assay<sup>22</sup>) yield estimates of the proportion of cytosines which are available for methylation but give no indication of how these cytosines are distributed across the genome. It is possible that

alterations in cellular folate status result in methylation changes within “junk” DNA regions with no obvious implications for genomic stability or for gene expression. More informative techniques are needed for mapping changes in genomic methylation in response to nutritional or other interventions. Possible approaches to this problem include developments of the enzymatic regional methylation assay<sup>23</sup> or exploitation of the power of pyrosequencing following bisulphite modification of DNA.<sup>24,25</sup> Characterisation of regional changes in DNA methylation patterns should be linked with investigations of expression of associated genes.

Although CRC is a focal disease arising from genomic damage to mitotically competent cells within individual crypts, it is widely assumed that there are “field” changes across large regions of the macroscopically normal mucosa which predispose to, or are biomarkers of the risk of, tumour development. This is the basis for attempts to develop surrogate end points which can be measured in pinch biopsies of colorectal mucosa.<sup>26</sup> Support for this concept comes from studies of crypt cell proliferation which demonstrate markedly different values between individuals but relatively low inter biopsy variation within subjects.<sup>27</sup> Before global DNA methylation can be accepted as a potential biomarker of bowel cancer risk it will be important to undertake studies of the heterogeneity of this putative marker between biopsies taken at the same site and between anatomical sites along the colorectum.

In respect of global DNA methylation, Pufulete’s observations<sup>16</sup> suggest that leucocytes may be a more easily obtained surrogate for colonocytes but we do not know how closely matched are baseline values or changes in response to supplementation in the two tissues. Exploitation of faeces as a non-invasive source of information about global DNA status in the colorectal mucosa is an obvious target following the recent demonstration that the methylation status of promoter regions of genes can be quantified in human DNA recovered from stool samples.<sup>28,29</sup> However, the huge preponderance of bacterial over human DNA in stool means that the commonly used techniques for assessing global DNA methylation will be inappropriate unless complete separation of the two DNA sources can be achieved or polymerase chain reaction based techniques are used to pull out specific human sequences.

In summary, observational studies in human populations together with investigations in animal models and cell

systems provide the evidence for the hypothesis that low folate intakes predispose to greater risks of CRC. Aberrant DNA methylation with its consequential effects on gene expression and on genomic stability offer a plausible causal link between cellular folate status and neoplastic development and therefore it will be important to build on the findings of Pufulete and colleagues<sup>16</sup> to determine optimal intakes of folate to minimise the risk of bowel cancer. Given the recent observation that high dose folate supplementation during pregnancy may increase the risk of death from breast cancer<sup>30</sup> it would be unwise to assume that, in respect of folate, more of a good thing is necessarily better.

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Correspondence to: Professor J C Mathers, Human Nutrition Research Centre, School of Clinical Medical Sciences, University of Newcastle, Newcastle upon Tyne, NE1 7RU, UK; john.mathers@ncl.ac.uk

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## Autoimmune pancreatitis

# Autoimmune pancreatitis—also a Western disease

R Sutton

Autoimmune pancreatitis is recognised worldwide as a T cell mediated, organ specific disease characterised by lymphocytic infiltration of the exocrine parenchyma with fibrosis and autoantibodies, including to carbonic anhydrase II. Increasing use of biopsy at endoscopic ultrasound may permit more frequent diagnosis without resection, but if doubt remains as to the presence of neoplasia, resection is preferable

During the five decades after Hashimoto described struma lymphomatosa of the thyroid gland featuring diffuse lymphocytic infiltration, fibrosis, and parenchymal atrophy,<sup>1</sup> autoimmunity became increasingly recognised, such that when similar histological changes were described affecting the pancreas, an autoimmune mechanism was postulated.<sup>2</sup> Autoimmune pancreatitis then remained among a number of plausible explanations for the appreciable minority of patients with chronic pancreatitis who give no history of alcohol abuse.<sup>3</sup> More recently, Japanese workers again took up the baton, describing series of patients who had undergone pancreatic resection for suspected neoplasia but whose histology demonstrated lymphocytic infiltration of the exocrine parenchyma, with periductular and interlobular fibrosis.<sup>4,5</sup> Further study demonstrated a T cell predominance to the infiltrate,<sup>5</sup> autoantibodies directed against carbonic anhydrase II,<sup>6</sup> an enzyme present in pancreatic as well as salivary ductal epithelium, and frequently high serum levels of IgG4.<sup>7</sup> These findings have been confirmed in

the European population, as reported by Aparisi *et al* in this issue of *Gut*,<sup>8</sup> who describe a small series within a larger group of patients with non-alcoholic chronic pancreatitis (*see page 703*). Although not stated, such a group is highly likely to include patients with mutations of the cystic fibrosis transmembrane conductance regulator (CFTR)<sup>9</sup> and serine protease inhibitor Kazal type 1 (SPINK1)<sup>10</sup> which both increase the risk of chronic pancreatitis, as well as mutations of cationic trypsinogen (protease, serine, 1 (trypsin 1): PRSS1) which cause hereditary pancreatitis.<sup>11</sup>

Confidence in the entity autoimmune pancreatitis is predicated not only on circumstantial clinical evidence that includes HLA restriction,<sup>12</sup> but also substantial indirect proof from animals that is perhaps as strong as for other autoimmune gastrointestinal diseases that include Sjögren's syndrome, achalasia, atrophic gastritis, pernicious anaemia, autoimmune hepatitis, primary biliary cirrhosis, sclerosing cholangitis, inflammatory bowel disease, and autoimmune gastroenteropathy.<sup>13</sup> Spontaneous development of autoimmune

pancreatitis is a characteristic of the autoimmune disease prone MRL/+ mouse<sup>14</sup> and Wistar Bonn/Kobori (WBN/Kob) rat,<sup>15</sup> and also occurs in German shepherd and rough coated collie dogs.<sup>16</sup> Neonatal thymus transplantation corrects the autoimmune pancreatitis and sialoadenitis of adult MRL/+ mice,<sup>17</sup> indicating a T cell dependent process. Adoptive transfer of CD4 (helper) T cells from MRL/+ mice with autoimmune pancreatitis into unaffected syngeneic animals induces autoimmune pancreatitis.<sup>14</sup> Autoimmune pancreatitis can also be induced by adoptive transfer of CD4 T cells from mice immunised by repeated administration of carbonic anhydrase II and/or lactoferrin in Freund's adjuvant.<sup>18</sup> Both CD4 and CD8 (suppressor/cytotoxic) T cells infiltrate pancreatic exocrine tissue in the MRL/+ mouse,<sup>14</sup> WBN/Kob rat,<sup>15</sup> German shepherd, rough coated collie,<sup>16</sup> and humans,<sup>5,19</sup> suggesting a mechanism akin to that in autoimmune beta cell destruction of the non-obese diabetic (NOD) mouse, BioBreeding/Worcester rat, and humans.<sup>20</sup> It has been suggested that diabetes mellitus complicating autoimmune pancreatitis may be due to bystander injury,<sup>5</sup> but the relatively large arterial flow to pancreatic islets and the widespread distribution of beta cells within islets would tend to minimise this; also, immune destruction of islets is highly specific.<sup>21</sup> Direct T cell mediated damage of beta cells in autoimmune pancreatitis is suggested by the presence of CD8 cells infiltrating islets in patients with the disease, in association with a diminished proportion of beta cells.<sup>22</sup>

The autoimmune spectrum extends from organ specific autoimmune disease, as in autoimmune thyroiditis and beta cell destruction causing type 1 diabetes mellitus, through intermediate forms such as primary biliary cirrhosis, to systemic autoimmune disease including systemic lupus erythematosus and rheumatoid arthritis, both of which can



feature organ specific autoimmune disease.<sup>20</sup> Patients with autoimmune pancreatitis may present with or develop Sjögren's syndrome, primary biliary cirrhosis, a form of sclerosing cholangitis, inflammatory bowel disease, as well as type 1 diabetes mellitus.<sup>5 19 22</sup> Local inflammation may extend to the gall bladder and duodenum<sup>23</sup>; furthermore, periportal and/or retroperitoneal fibrosis<sup>19</sup> may complicate the picture, perhaps because of determinant spreading, broadening the antigenic targets of autoimmunity.<sup>20</sup> At the other end of the spectrum, systemic lupus erythematosus has been described featuring both acute<sup>24</sup> and chronic<sup>25</sup> pancreatitis not attributable to drugs. Systemic autoimmunity may have a somewhat different pathogenesis, with damage initiated by antibody and complement responses to multiple products of apoptosis<sup>20</sup>; the pancreas, therefore, may be subject to more than one form of autoimmune attack.

Interestingly, an autoimmune component in acute pancreatitis has increasing been recognised.<sup>26</sup> Cases of autoimmune pancreatitis now on record include patients presenting with focal as well as more recognisably typical diffuse pancreatic masses, and occasionally pseudocysts, parenchymal atrophy, and/or calcification.<sup>5</sup> These latter apparently atypical findings have been used by some to exclude the diagnosis,<sup>19</sup> as in the current study by Aparisi and colleagues,<sup>8</sup> who also separated patients with Sjögren's syndrome from those with suspected autoimmune pancreatitis. While this approach has some specificity, it does not have sensitivity; consensus on diagnostic criteria is yet to be achieved. As with other forms of pancreatic injury extending over prolonged periods, there is likely to be a range of clinical findings that includes acute as well as the more prevalent chronic forms, the full extent of which awaits complete characterisation. Further unrecognised autoimmune pancreatitis may be present in patients with other autoimmune diseases where the condition is not considered, or the predominant symptoms are not pancreatic, or where treatment with steroids or other immunosuppressive agents masks or alters the progression of autoimmune pancreatitis.

Increasing acceptance of autoimmune pathology affecting the exocrine pancreas has prompted a call for the term autoimmune pancreatitis<sup>27</sup> to replace the obscure terms sclerosing pancreatitis, primary inflammatory pancreatitis, lymphoplasmacytic pancreatitis, sclerosing pancreaticocholangitis, idiopathic tumefactive chronic pancreatitis, lymphoplasmacytic sclerosing pancreatitis,

and idiopathic duct destructive pancreatitis.<sup>28</sup> For clarity we would diagnose all cases as autoimmune pancreatitis without differentiation of "primary" from "secondary" forms depending on the absence or presence of other autoimmune disease,<sup>27</sup> because the association with other autoimmune disease has yet to be fully defined, and when present with other autoimmune disease, the pathogenetic mechanism is not necessarily "secondary". It is of interest to reflect on the parallels with autoimmune thyroiditis, a condition that had many names, including chronic thyroiditis, lymphocytic thyroiditis, lymphadenoid goitre, and the original struma lymphomatosa,<sup>1</sup> which for some time was a diagnosis made on the pathological assessment of resected specimens; notably, the condition used to be considered an uncommon disease.

Autoimmune pancreatitis can present with a non-specific history of pain and/or anorexia with weight loss that is often accompanied by jaundice from biliary obstruction; the condition may be heralded by an initial attack of acute pancreatitis.<sup>23</sup> Other autoimmune diseases of either the patient and/or family should draw attention to the possible diagnosis. As well as, or as an alternative to, elevated levels of IgG4 and positive anticarbonic anhydrase II titres, antilactoferrin, antinuclear, antismooth muscle, and rheumatoid factor antibodies may be identifiable.<sup>5</sup> Germline gene analysis must also be undertaken to identify patients with CFTR, SPINK1, or PRSS1 mutations,<sup>9 10 11</sup> as there is overlap in the clinical features associated with all forms of chronic pancreatitis.<sup>3 11</sup> Radiological imaging by computerised tomography (CT), magnetic resonance imaging (MRI), or endoscopic ultrasound (EUS) may show a sausage shaped swollen pancreas or focal masses that can be single or even multiple.<sup>29</sup> Endoscopic retrograde cholangiopancreatography (ERCP) may identify biliary strictures from pancreatic compression or a pattern typical of sclerosing cholangitis, as well as pancreatic ductal stenoses<sup>29</sup>; stenting may be required. The presentation can be indistinguishable from that of pancreatic cancer or much rarer pancreatic lymphoma, which should be considered for resection and/or chemotherapy. Transduodenal EUS guided biopsy offers the best prospect for unravelling this conundrum,<sup>5 27</sup> but it must be emphasised that a trial of steroid therapy cannot be recommended if substantial doubt remains as to the diagnosis in an operable patient. Such a trial of treatment could reduce inflammation surrounding a cancer and be interpreted as appropriate, only to be followed

by progression to unresectability. In any case, optimal treatment regimens are undefined and a morphological response may take a number of weeks if not months<sup>5 19</sup>; currently, resection is the commonest route to diagnosis. While recognising the importance of surgery when indications remain, and the acceptable results of surgery in tertiary centres when autoimmune pancreatitis is the final diagnosis,<sup>3 30</sup> recent experience at the Mayo Clinic has been that of a diagnosis of autoimmune pancreatitis in an increasing number of patients, with a decreasing percentage of patients undergoing surgery (Dr S Chari, personal communication, 2004). Increasing recognition of this disease has led to the identification of more cases where there is little or no indication for surgery but some cases may have to be teased out from an otherwise appropriate surgical pathway.

It is recommended that all patients with a possible or suspected diagnosis of autoimmune pancreatitis be referred to a pancreatologist, usually at a regional pancreas centre. This recommendation is all the more important as there are no published guidelines for the management of this condition. If surgery is deemed inappropriate, pancreatic exocrine and endocrine function should be formally assessed at an early stage. Suggestions as to therapy are preliminary, but if there is justifiable confidence in the diagnosis and symptoms significant, a reducing dose of prednisolone is reasonable. With this regimen, resolution of pancreatic pain as well as exocrine and endocrine insufficiency has been seen; partial or complete resolution of biliary strictures of an apparently atypical form of sclerosing cholangitis has been witnessed by us and an increasing number of workers.<sup>5 29</sup> Supplementary exogenous pancreatic enzyme supplementation may be required for some weeks, months or indefinitely, while treatment for diabetes mellitus will require joint care with a diabetologist; rheumatological or other expertise may also be required to help tailor treatment in patients with other autoimmune disease. Measures to monitor treatment response include assessment of symptoms, pancreatic exocrine and endocrine function, as well as full blood count, liver function tests, EUS with or without biopsy, CT, or MRI; in patients with endoprostheses, long term antibiotics may be justified and a further ERCP will be needed to remove the stent at an early opportunity. How long to continue steroids will depend on the response, side effects, and the effect of treatment withdrawal, as well as the presence and need for treatment of other autoimmune disease. Whether

there is a role for alternative therapies remains to be defined, as does the place of autoimmune pancreatitis in the gamut of autoimmune diseases.

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Correspondence to: Professor R Sutton, Division of Surgery and Oncology, University of Liverpool, 5th Floor UCD Block, Royal Liverpool University Hospital, Daulby St, Liverpool L69 3GA, UK; r.sutton@liv.ac.uk

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