REVIEW

Micronutrient Therapy for Chronic Pancreatitis: Rationale and Impact

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Summary

Micronutrient therapy, designed to buttress tissue methyl and thiol groups, curbs attacks and controls background pain in patients with chronic pancreatitis, irrespective of aetiology. This outcome and the premises upon which it is based facilitate an understanding of links with mutations in genes for hereditary pancreatitis and cystic fibrosis, demography, and predisposition to pancreatic cancer. Above all, there is an opportunity for prophylaxis in individuals at high risk of developing the disease.

Introduction

It is a sobering thought that more than 200 years after the post-mortem identification of chronic pancreatitis [1], removal of the whole gland is an accepted form of treatment when pain is unrelenting [2]. Micronutrient therapy, formulated from observations at Manchester over a 15-year period 1983-1998 [3, 4, 5, 6, 7, 8, 9], is poised to change this bleak outlook now that a definitive study from Delhi has been published [10], after trials in Santander [11], Belfast [12], Brno [13] and Naples [14]. This paper sets out the background to the treatment and expands on the implications of its success, in the light of groundbreaking advances in genetic information.

The Disease

Natural History

Chronic pancreatitis is a crippling disease. It maims by increasingly agonizing pain with superimposed attacks

of pancreatitis on a variable timescale [15]. Each bout causes patchy loss of acini until all are destroyed and replaced by fibrous tissue, when pain tends to regress. By then, however, patients have often become addicted to narcotic analgesics, lost their jobs, homes and families. Duct strictures and calculi may develop and are readily diagnosed, in contrast to small-duct disease [4]. Pancreatic cancer looms [16]. Most patients fall into alcoholic and idiopathic groups, the latter with its tropical variant. Among the others hereditary pancreatitis, autoimmune disease, hyperlipidaemia or hyperparathyroidism may be detected. Alcohol is over-rated, in that about 18 years elapse before the first symptom in men who drink more than 150 grams of ethanol daily. Any theory on pathogenesis should reconcile the paradox that some fall prey after just 20 grams per day, and other idiosyncrasies; accommodate genetic links; and rationalize demography [15].

Pathopysiology: Initiating Mechanisms

Today acute pancreatitis which is characterized by a histological *restitutio in integrum* after full clinical recovery, recurrent acute pancreatitis, and the irreversible inflammatory fibrosis of chronic pancreatitis are regarded more as a disease continuum than utterly different entities [15, 16]. This view is supported by the clinical and biochemical identity of a pancreatitis attack, overlapping extrinsic [3, 4] and genetic [16] aetiologies, and the observation that experimental protocols which induce acute pancreatitis can be manipulated to cause fibrosis [15, 17].

It has long been known that experimental pancreatitis begins in the acinar cell with a functional blockade to apical exocytosis of enzymes [18], for which anomaly the term 'pancreastasis' seems apt [17]. Subsequent events can be interpreted as a strategy to prevent the

build-up of food digestive enzymes, when the cell perceives a threat to its secretory integrity [17].

i) Newly synthesized enzyme is quickly shunted out via the basolateral membrane into the portal venous outflow and lymphatics [19], which could account for the blood enzyme rise in a clinical attack of pancreatitis.

ii) Stored enzyme is removed by centripetal dissolution of zymogen granules [20] and by their redirection to the basolateral membrane [21].

iii) A controlled activation of trypsinogen in the secretory pathway by co-localization with lysosomal enzymes, which yields <2% of potential trypsin load [22] and facilitates protease degradation [20]; and

iv) apoptosis of cells that contain trypsin or mutated cationic trypsinogen [23] could be part of the same strategy.

Alternatively, as is now generally believed, the co-localization phenomenon is pathological and signifies the start of pancreatic 'autodigestion' [16].

Whatever the true interpretation may be, time-course analysis shows that a burst of electron transfer reactions is tied in with the disease-initiating secretory blockade [17]. For example, in the experimental model of mild acute pancreatitis produced by excessive stimulation with caerulein, the spark from reactive oxygen species is seen by chemiluminescence within five minutes as also is a huge increase in stress activated protein kinase evoked thereby: amylase in the gland's venous outflow increases by 10 minutes in line with the reversed secretory polarity [24]. Similarly in endoscopic retrograde cholangiopancreatography (ERCP)-induced acute pancreatitis, analysis of peripheral blood by electron spin resonance spectroscopy identifies the burst of reactive oxygen species by the end of the clinical procedure, followed by steep increases in amylase, lipase and trypsinogen [25].

The complex downstream interactions that result in pancreatic inflammation have been reviewed [26]. They involve reactive oxygen and nitrogen species, free radical oxidation products, chemokines and cytokines liberated by a variety of cells, including the injured acinar cell.

Chronic Pancreatitis: Casualty of Oxidative Detoxification

Manchester Model

This disease model has evolved over the years to accommodate new observations. The 1998 version [27] views the acinar cell as the site of steady erosion of methyl and thiol, essentially glutathione, moieties as a result of regular exposure to xenobiotics that induce cytochrome P450 (CYP) mono-oxygenases while yielding electrophilic intermediates: inadequate prior diets set the scene. Within this framework, each burst of unopposed electron transfer reactions jeopardizes

apical exocytosis to trigger an attack. The secretory diversion drives pro-inflammatory oxidation products into the interstitium, such that mast cells degranulate: this leads to fat necrosis, activation of the 'contact system' of blood coagulation, nociceptive axon reflexes, and profibrotic interactions. Duct cells are caught up in a manner that unfortunately amplifies the disease. These problems are compounded when relatively stable substances generated via induced hepatic CYP find their way into the gland by bile reflux or the bloodstream [15, 17]. The template allows for methyl-thiol depletion due to protracted stress from reactive oxygen species alone, as in hereditary pancreatitis (see below). In regard to autoimmune disease, wherein lactoferrin is a suspected antigen [28], the ability of reactive oxygen species to derange the structure and hence immunogenicity of gamma-globulin is relevant.

CYP Induction; Toxic Electrophiles

The metabolism of xenobiotics in the liver is usually a two-stage process [29]. In phase-1, microsomal CYP utilize reactive oxygen species to reveal or insert a functional group such that the intermediate metabolite can move into the cytoplasm for phase-2 conjugation reactions with glucuronic acid, inorganic sulphur, acetyl groups or glutathione. In the process the substrate is usually detoxified, or de-activated in the case of a drug. Regular exposure to a xenobiotic results in an increase of the corresponding CYP isoform, as can be gauged by studying the disposal of drug probes: for example, theophylline clearance kinetics reflect activity of CYP1A that is induced by polycyclic aromatic and chlorinated hydrocarbons [29]. This protective mechanism of enzyme induction backfires, however, if a xenobiotic inadvertently undergoes bioactivation to a hazardous intermediate metabolite, for now cell viability is threatened both by the increased load of reactive oxygen species and by reactive xenobiotic species: the former is especially true for induction of CYP2E1 [30]. The concerted action of superoxide dismutase in two forms (manganese or copper-dependant), catalase, glutathione peroxidase, and pyridine nucleotides limits the lifespan of reactive oxygen species [31]. Glutathione, synthesized from the essential aminoacid methionine (Figure 1), is the main defence to reactive xenobiotic species but, whereas it is easily regenerated after reactions in removing oxygen free radicals, it is irretrievably lost in glutathione transferase-mediated conjugation with reactive xenobiotic metabolites [31, 32]. Clinicians are familiar with these principles in relation to paracetamol hepatotoxicity.

High fat/protein diets, as are associated with chronic pancreatitis, facilitate CYP induction: constituents of cigarette smoke, which increase disease risk, are potent inducers and generate reactive intermediates [3]. The bulk of ethanol is processed oxidatively via the alcohol-acetaldehyde route; there is a non-oxidative route that yields free fatty acid ethyl esters; both have

been implicated in pancreatic injury [33, 34]; and duct cells are active too [35] - but long-term ethanol does not produce chronic pancreatitis experimentally [36]. However, even a small dose induces CYP2E1, thus increasing the toxicity from other chemicals to which the animal is simultaneously exposed [37]. This becomes relevant with the identification of occupational volatile chemicals (diesel exhaust fumes and chlorinated solvents in particular) as an independent risk factor for chronic pancreatitis at Manchester [38], as also of the association with the disease of those chemicals alongside domestic paraffin at Soweto [33, 39] or domestic kerosene at Madras (Chenai) [40].

A large-scale pharmacokinetic investigation indicated CYP induction, especially CYP1A, in the majority of Manchester patients with chronic pancreatitis [41].
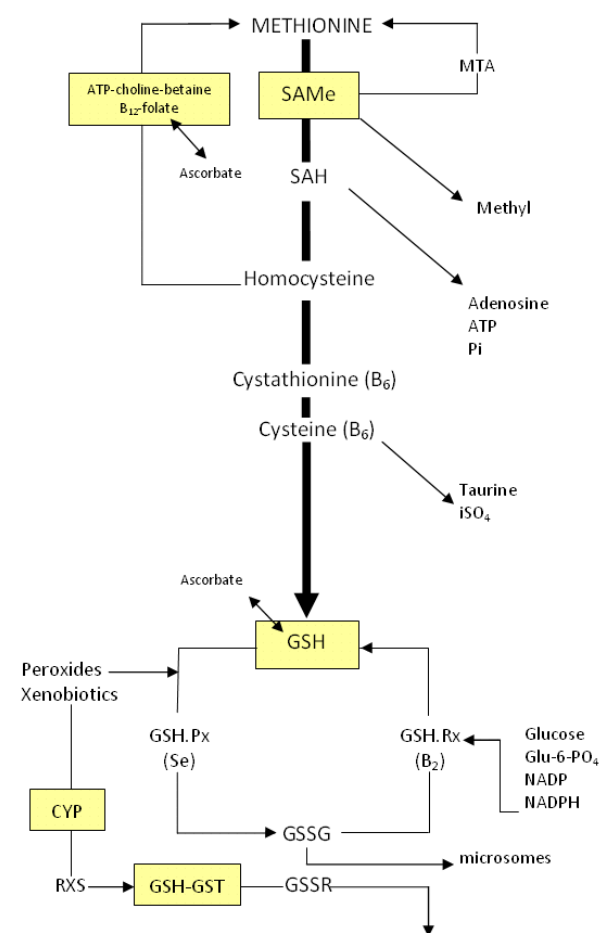

Figure 1. Methionine metabolism, adapted from [15]. Abbreviations are listed alphabetically. ATP: adenosine triphosphate; B_6: pyridoxyl-dependant enzymes; B_{12}: cyanocobalamin; CYP: cytochromes P450; iSO_4: inorganic sulphate; glu-6-PO_4: glucose-6-phosphate; GSH: glutathione; GSH-GST: the glutathione-glutathione transferase couple; GSH.Px: selenoenzyme glutathione peroxidase; GSH.Rx: riboflavin-dependant glutathione reductase that interlinks with shuttles involving glucose-6-phosphate (glu-6-PO_4) and nicotine adenosine phosphate in oxidized (NADP) or reduced (NADPH) form; GSSG: reversibly oxidized form of glutathione; GSSR: irreversibly oxidized form of glutathione that is excreted from the cell; MTA: methyl thioadenosine; Pi: activated phosphate; RXS: reactive xenobiotic species; SAH: sulph-adenosylhomocysteine; SAMe: sulph-adenosylmethionine.

Proof of acinar involvement has come from immunolocalization studies, which also show a degree of induction in ductal elements [42, 43, 44]. Although induced pancreatic CYP contribute virtually nothing to overall xenobiotic removal [29, 43], their toxicological significance is evident from structural and biochemical analysis: cytoplasmic vacuolation and excess lipofuscin in acinar cells [4, 15, 17]; dilated endoplasmic reticulum and increased lysosomes in duct cells [4]; increased concentrations of free radical oxidation products in the gland [45], pancreatic juice [46] and duodenal aspirates [5, 47]; mobilization of endogenous antioxidant defences [48] such as lactoferrin by acinar cells [4, 28] and mucus by ducts [40].

The CYP-induced hepatocyte is under strain too, as revealed by microvesicular steatosis and excess lipofuscin in many non-alcoholic patients [15]: increased bilirubin in bile, with a striking surge during a relapse, reflects the activation of haem oxygenase and both represent antioxidant defences [15, 48]. Yet these aberrations are silent, while the acinar cell bears clinical witness to the xenobiotic assault. This is analogous to the far greater sensitivity of the pancreas to CYP-mediated injury from carbon tetrachloride [17, 27]. Also of note, a single injection of dibutyltin (which has many industrial applications [32]) produces within 60 days a good animal model of chronic pancreatitis after an initial phase of acute inflammation, with damage amplification by doses of ethanol that are otherwise harmless, indicating CYP2E1-mediated reactive xenobiotic metabolites [49]. Several factors help to rationalize these findings.

i) There is a dearth of glutathione transferases in acinar cells [32, 42, 50, 51] and virtually no copper-superoxide dismutase [52] - whereas duct cells are better protected - while pancreatic levels of the other antioxidant enzymes fall in chronic pancreatitis [53].

ii) The pancreas has a much lower complement of glutathione than the liver [32].

iii) Its pool of cysteine for glutathione synthesis is small, and needed for protein folding [32].

iv) The inhalation route of xenobiotic entry [33, 38, 39, 40] ensures a direct arterial strike once the pulmonary circulation is traversed.

v) Although islets have a generous quota of glutathione transferases [42, 50, 51], they show such potent CYP induction in chronic pancreatitis [42, 43, 44] that reactive xenobiotic metabolites which escape into the gland's portal circulation would amplify injury [27, 51].

vi) Above all, considering the huge turnover of protease grenades, is the vulnerability to toxic electrophiles of the signal transduction pathway towards apical exocytosis [15, 18, 54].

Disrupted Methyl and Thiol Metabolism

Toxic electrophiles can disrupt signal transduction in many ways [17, 27], and it may be that different

mechanisms operate in different settings of acute pancreatitis. However, reviewed studies in young female mice with lethal disease due to a choline-deficient DL-ethionine-supplemented diet offer useful insights [17, 55]: the relative safety of older females but vulnerability of oestrogen-treated males implicates a reactive oestrogen metabolite [17]. A burst of electron transfer reactions is detected by electron spin resonance spectroscopy at six hours, and apical secretion is paralysed by 24 hours with death by the fifth day. DL-ethionine injury is caused by a metabolic blockade high up in the methionine trans-sulphuration route, such that the supply of adenosine for synthesis of adenosine triphosphate, and of both methyl and thiol groups is compromised: denial of choline augments these problems (Figure 1). Of note, methyl and thiol (glutathione) moieties are essential for exocytosis [17, 27], as is folate which acts as a methyl donor [55]. Reports on paracetamol and carbon tetrachloride toxicity show that key enzymes in the trans-sulphuration pathway are vulnerable to reactive xenobiotic metabolites [15, 27]. Modification of the DL-ethionine regimen induces chronic pancreatitis-like lesions [17, 55].

Studies of human chronic pancreatitis indicate a similar pattern. Thus, at admission in an attack, neutrophils show low glutathione but increase in the oxidized disulphide form indicating electrophilic stress [56], while urine [56] and blood analysis [7] point to a metabolic block in the trans-sulphuration pathway, but now distal to cysteine (Figure 1) - leading to surges in cysteine and more proximate metabolites, but a fall in inorganic sulphur. By the third day subnormal methionine and a further decline in sulphur levels hint at poor pre-morbid intakes. These twin problems, of on-going oxidant-related hindrance in the trans-sulphuration pathway and methionine insufficiency, are reflected in findings on serum/plasma analysis in quiescent disease: increased free radical oxidation products [7, 8, 10, 33, 45, 57, 58] but subnormal methionine (unpublished), sulph-adenosylmethionine [7], cysteine and cysteinyl glycine [57], taurine [59], total thiols [58] and glutathione [33, 57]. Moreover, a study using radiolabelled L-methionine showed good uptake by the pancreas but then backdiffusion, coupled with reduced enzyme and label secretion into the duodenum [60]. In these conditions cysteine appears to be diverted along pathways that generate hydrogen sulphide [61], which is increased in exhaled air of patients with chronic pancreatitis [62] and has been linked experimentally to pancreatic pain [63].

Genetic Studies

The possibility of a genetic predisposition to chronic pancreatitis due to heightened oxidative metabolism or defective antioxidant/conjugation resources has been explored but without consistent evidence for either [34, 64, 65]. However, two reports on idiopathic disease suggest susceptibility to xenobiotic stress: increased frequency of functional glutathione transferase form *GSTT1*A*, which tends to yield reactive xenobiotic metabolites [26], but lowered glutathione and its redox ratio in erythrocytes [64]; increased frequency of the *PON1-192Q* allele of paraoxonase which compromises its xenobiotic detoxification capability [66].

Table 1. Clinical trials of micronutrient therapy in chronic pancreatitis.

Author and location	Clinical details	Trial type and active treatment
Uden, Manchester [6, 7] [a]	20 patients ACP: n=7; ICP: n=8; RAP: n=5 Steatorrhoea: none	20-week switchover double-dummy double-blind Active treatment: 6/day selenium-beta-caroteneCE (Wassen); 8/day methionine (Evans) Daily dose: Se 600 µg, beta-carotene 54 mg, C 540 mg, E 280 mg, methionine 2 g
Bilton, Manchester [8] [a b]	20 patients ACP: n=2, ICP: n=10, RAP: n=8 Steatorrhoea: none	20-week switchover double-dummy double-blind Active treatment: 6/day sulph-adenosylmethionine (Bioresearch) Daily dose: 2.4 g
Kirk, Belfast [12]	19 patients All ACP (?) Steatorrhoea (?)	20-week switchover placebo-controlled double-blind Active treatment: 4/day (Antox®) Daily dose: Se 300 µg, beta-carotene 12 mg, C 600 mg, E 188 mg, methionine 1.6 g
De las Heras Castano, Santander [11]	19 patients ACP: n=11, ICP: n=5, RAP: n=3 Steatorrhoea (?)	Open trial for 12 months Active treatment: 4/day (hospital preparation) Daily dose: Se 300 µg, beta-carotene 12 mg, C 600 mg, E 188 mg, methionine 1.6 g
Ditě Brno [13] [c]	70 patients ACP: n=59; ICP: n=3, other: n=8 ERCP grade: 1: n=16; 2: n=24; 3: n=30 Steatorrhoea: 80% of grade 3	Open trial for 12 months Active treatment: 1/day C and 1/day E Daily dose: C 500 mg, E 100 mg
Bhardwaj, Delhi [10]	127 patients Study power: 80% ACP: n=35; ICP: n=92 ERCP grade (?): 1: n=22%; 2-3: n=78% Steatorrhoea: 20%	6-month parallel placebo or active Active treatment: (?)/day (Betamore G®) Daily dose: the same as Manchester 1990 [6] Plus all on pancreatic enzyme supplements
Uomo, Naples [14]	3 patients Children with hereditary pancreatitis	2-year open in 4 blocks of 6 months; active treatment at 2nd and 4th block Active treatment: 2/day sulph-adenosylmethionine (Bioresearch), 3/day multivitamin (?) Daily dose: Se 75 µg, A 2.4 g, C 180 mg, Mg 300 mg, E 30 mg, SAMe 800 mg

Continues

Table 1. Continued.

Author and location	Outcome	Biochemistry
Uden, Manchester [6, 7] [a]	Attacks ↓ 11 pain words VAS ↓ Pain diaries 2nd 5-wk period ↓ Pain psychology ↔ Placebo effect	Baseline Se and beta-carotene < controls, E and A ↔; post-Tx all ↑↑ Baseline SAMe < controls; downward drift post-Tx Baseline MRLA > controls; post-Tx ↓ Baseline pGSH.Px and uSO$_4$ = controls; post-Tx ↔ pSAMe in attack ↑; Se *vs.* MRLA nomogram useful No carry-over at 10 week
Bilton, Manchester [8] [a b]	Attacks ↔ Words VAS ↔ Pain diaries ↔ (Diarrhoea)	Baseline Se, beta-carotene, AA and C < controls; post-Tx ↔ Baseline % C oxidized > controls; post-Tx ↔ Baseline E and A = controls; post-Tx ↔ Baseline MRLA > controls; post-Tx ↔ Baseline SAMe = controls; post-Tx ↑↑
Kirk, Belfast [12]	SF-36 quality of life score ↑ Pain ↓ Physical/social well-being ↑ (Nausea)	No control data; post-Tx Se, beta-carotene, C and E > placebo Post-Tx A, alpha-carotene and TAC ↔ Post-Tx Fox1, MDA, and pGSH ↔ Carry-over at 10 week
De las Heras Castano, Santander [11]	Admissions vs previous year ↓ Pain word VAS vs previous year ↓ Pancreatic function vs previous year ↔	Not done
Dítĕ Brno [13] [c]	Melzack pain scale ↓↓: Pain in 44% abolished Pain in 56% ↓↓ Analgesics ↓↓ Post-Tx: grade 2 ERCP ↔	Pre-Tx ROS generation varies as 1/ERCP grade Pre-Tx lipid peroxides varies as 1/ERCP grade Pre-Tx C and E < controls, vary directly as ERCP grade Post-Tx C ↑, highest in ERCP grade 3 Post-Tx E ↔ Post-Tx ROS generation ↓ in ERCP grades 1 and 2; not 3
Bhardwaj, Delhi [10]	Admissions ↓, 32% pain-free Pain days/month ↓ Analgesics ↓, man-days lost ↓ Improvement by 3 months Placebo effect, e.g. 13% pain free (Headache, constipation)	Baseline A and E < controls, C = controls Baseline FRAP,E-TGSH & E-SOD < controls, Post-Tx all these ↑, vitamins by 1 month Baseline S-SOD & TBARS > controls, post-Tx ↓ 3-month data not given
Uomo, Naples [14]	Painful days ↓ Analgesics ↓	Not done

[a] Retinol data not cited but normal

[b] Trial of sulph-adenosylmethionine together with selenium-beta-carotene also reported but abandoned when post-trial patients had attacks on active treatment

[c] Data on vitamin C as personal communication (mean±SD): controls, 3.62±0.81 mg/L; grade 1 ERCP (endoscopic retrograde cholangiopancreatography), 2.54±0.41 mg/L; grade 2, 0.89±0.52 mg/L; grade3, 0.71±0.23 mg/L

Arrows signify direction and degree of change. Question marks (?) indicate information not given. Abbreviations are listed alphabetically. A: retinol; AA: ascorbic acid; ACP: alcoholic chronic pancreatitis; C: vitamin C; E: vitamin E; E-SOD: erythrocyte superoxide dismutase; E-TGSH; erythrocyte total glutathione; Fox1: ferrous oxidation; FRAP :free radical trapping ability of plasma; ICP: idiopathic chronic pancreatitis; MDA: malondialdehyde; Mg: magnesium; MRLA: molar ratio of linoleic acid isomer to parent acid; pGSH: plasma glutathionine; RAP: recurrent acute pancreatitis; ROS: reactive oxygen species; S-SOD: serum superoxide dismutase; SAMe: sulph-adenosylmethionine; Se: selenium; VAS: visual analogue score; *vs.*: *versus*; TAC total antioxidant capacity; TBARS thiobarbituric acid reacting products of lipid peroxidation; Tx: active treatment; uSO$_4$: urinary inorganic sulphate. Trade names: Antox® (Pharma Nord Ltd., Northumberland, United Kingdom); Betamore G® (Osper Pharmaceuticals, Delhi, India). US approved names: methionine (Evans Medical Ltd., Horsham, United Kingdom). Other active treatments: selenium-beta-caroteneCE (Wassen International Ltd., Leatherhead, United Kingdom); sulph-adenosylmethionine (Bioresearch, Milan, Italy).

Micronutrient Therapy

Clinical Trials

Several micronutrients have antioxidant potential [31]. However, a comparison of the habitual diets of patients with idiopathic chronic pancreatitis, an equally CYP1A-induced set of epilepsy controls on anticonvulsants, and healthy volunteers at Manchester identified the patients' need of just three (methionine, vitamin C and selenium [67]), which together should buttress pancreatic methyl and thiol groups (Figure 1). Subnormal serum/plasma concentrations of these micronutrients have been reported [7, 8, 33, 58, 68, 69, 70, 71] but are also influenced by increased oxidation, utilization, tissue sequestration, and malabsorption. In the 1980s there was no tablet that would deliver these, and only these, three substances. By trial and error,

using Selenium-ACE® (selenium with vitamins A, C and E; Wassen International Ltd., Leatherhead, United Kingdom) with or without methionine tablets (Evans Medical Ltd., Horsham, United Kingdom), we found that doses for symptom relief varied widely and that patients with recurrent acute pancreatitis benefited too: they displayed elevated levels of free radical oxidation products in duodenal aspirates and serum [5], although blood levels of micronutrients were generally normal [15, 70]. By 1985, observations in 23 patients showed that six Selenium-ACE® plus eight methionine tablets per day most often controlled symptoms [9]. That combination, but with beta-carotene in place of vitamin A, was selected for formal assessment. Table 1 summarizes results of trials to date, wherein every effort was made to eliminate bias [72].

Some interesting points emerge.

i) Treatment curbs attacks and controls background pain in chronic pancreatitis *pari passu* with a fall in markers of electrophilic stress and restoration of erythrocyte glutathione. This holds true irrespective of aetiology or pancreatogram appearance.

ii) The Dehli study shows that blood antioxidant profiles improve within a month and pain is controlled by three months, in keeping with the Manchester and Belfast experience at 10 weeks.

iii) The Brno study suggests that the degree of electrophilic stress is highest in patients with the greatest amount of functional parenecyhma, that is with least abnormal pancreatograms.

iv) Measures of total antioxidant status do not help to monitor micronutrient therapy, as the Belfast study shows for a kit method which largely reflects albumin, glucose, uric acid and bilirubin [73]. Similar problems beset the 'free radical trapping ability of plasma' assay as used at Delhi: ascorbic acid (the active form of vitamin C) normally contributes 20% to the reading, vitamin E 5%, beta-carotene very little and thiols none. The assay value in Delhi controls is much less than elsewhere [57, 74] although the endogenous component was normal (uric acid not reported) and plasma 'total vitamin C' as high as at Manchester, on average 12 mg/L [8]; further, the lower reading in patients improved on treatment although their baseline vitamin C level was normal. The first anomaly may be methodological [74]. The second strongly suggests low ascorbic acid in Delhi controls with a further fall in chronic pancreatitis, but masked in the total vitamin C assay - as has been noted previously [8, 33], including in a report from Madras which suggested ascorbate oxidation during cooking [75].

v) Studies of acute pancreatitis in cyclosporine-treated pancreatic allografts [76] suggested that sulph-adenosylmethionine may be enough (Figure 1). It was not, whereas combined treatment attenuated damage in a cyclosporine-hyperstimulation model of pancreatic fibrosis [77].

vi) We did not think that vitamin C alone as in the Brno trial in alcoholic chronic pancreatitis could work (vitamin E was given too, but its levels did not improve). However, there are experimental precedents. Thus: a synthetic analogue of ascorbic acid improved survival in the virulent dietary model of acute pancreatitis [78], and was very helpful in the caerulein model [79]; ascorbic acid ameliorated damage from dibutyltin [80]; and it attenuated the secretory blockade caused by the potent oxidant tert-butylhydroxyperoxide [81]. Several properties of ascorbic acid rationalize the benefit: its excellent capacity to scavenge electrophiles [82, 83]; its action as 'Michael donor' in toxicity studies of acrolein [82], which is a derivative of acetaldehyde, as well as in reactions with genotoxic lipid peroxidation products [83]; and redox coupling between glutathione/glutathione disulphide and ascorbic/dehydroascobic acid (Figure 1) [84]. The last

effect and the delayed onset of scurvy by treatment with glutathione-ester in ascorbate deficient guinea pigs [85] indicate metabolic substitution between ascorbic acid and glutathione. Since plasma vitamin C was very low in Brno controls (Table 1), the inference may be that methionine status was good, and that prescribed ascorbic acid in the chronic pancreatitis group protected trans-sulphuration enzymes from reactive chemical species.

The restoration of methyl and thiol groups needed for apical exocytosis in the acinar cell is the best explanation for the fall in attack frequency on micronutrient therapy. Lowered delivery of pro-inflammatory substances via basolateral pathways and increased micronutrient levels in tissues rationalize alleviation of background pain. Among the many factors implicated [86], these could be mollified by 10 weeks' treatment: increased interstitial fluid pressure; the inflammation-immune response [26, 87]; mast cells [17, 25, 27, 88] with the pro-inflammatory [17, 25, 89], and painful [27, 90] consequences of their activation; and the hydrogen sulphide effect on nociceptive channels [61, 63].

Trials of other electrophile scavengers in alcoholic chronic pancreatitis include a Baghdad study which noted dramatic benefit from daily intra-rectal treatment with allopurinol or dimethyl sulphoxide in an attack [91], and an anecdotal report of benefit after intravenous chlorophyll-A [92]. By contrast, trials in quiescent chronic pancreatitis showed that neither allopurinol [93] nor curcumin [94] alleviated pain. We conclude that the first two interventions controlled neutrophil-derived reactive oxygen species during exacerbations [95] but the others failed to protect the trans-sulphuration pathway in stable disease. It seems that a grape seed extract with potent antioxidant qualities was able to do so, judging by another anecdotal report [96].

Follow-up: Need To Control Reductive Stress?

The long-term value of micronutrient therapy at Manchester and practicalities of delivery have been documented [9, 97]. Among the 10% of treatment failures there were two patients (chronic pancreatitis and polycystic kidneys; recurrent acute pancreatitis and arthritis on 'sulindac') in whom a choline supplement to provide more methyl groups (Figure 1) afforded rapid relief (Braganza, unpublished). This benefit concurs with a shift in thinking, namely, that 'reductive stress' is the common cause of reactive oxygen species generation in human pathology (Dormandy unpublished, acknowledged in reference [98]). There is a growing body of indirect supportive evidence for this [98, 99]. Reductive stress can be simplistically envisaged as an increase in 'electron pressure' just as acidosis is an increase in 'proton pressure': the two are in fact closely linked (Dormandy, personal opinion, unpublished). The body's answer to reductive stress is probably to liberate labile methyl groups and generate methane which is exhaled, while ascorbic acid acts as

counterpoise by generating carbon dioxide in reactions with hydrogen peroxide in the presence of iron [99]. Excess alcohol, ischaemia-reperfusion, redox cycling drugs, and uncouplers like non-steroidal anti-inflammatory drugs and cyclosporine generate reductive stress. This is alleviated pharmacologically by electrophilic methyl groups as in choline [98], the prescription of which might circumvent the adverse effects, albeit theoretical, of prolonged methionine supplementation [87].

Corollary 1: Accommodating Genetic Links

Hereditary Pancreatitis

The large volume of genetic information has been reviewed twice in 2009 [100, 101]. There are six basic messages.

i) Gain-of-function mutations in the *PRSS1* gene that encodes for cationic trypsinogen cause hereditary pancreatitis and are transmitted in autosomal dominant fashion, with 80% penetrance.

ii) *PRSS1* mutations play little part in other forms of chronic pancreatitis.

iii) Loss-of-function mutations in the *SPINK1* gene that encodes for serine protease inhibitor Kazal type 1 are strongly associated with idiopathic chronic pancreatitis, especially the tropical variant, but are seen as disease-modifying, not causative.

iv) The last point is also true for mutation in the *CTRC* gene that encodes for chymotrypsinogen, the active form of which degrades trypsin.

v) Genetic links with alcoholic chronic pancreatitis are modest.

vi) About 50% of patients have normal trypsin-related genes and also *CFTR* (see below).

The potent inhibitory effect of thiols on trypsin and other proteases is overlooked by proponents of the theory that mutations in trypsin-related genes are synonymous with pancreatic autodigestion [16]. The crucial mechanism of serine protease control by thiols was described 40 years ago and elucidated by Manchester scientists in elegant *in-vitro* studies [102, 103]. They showed that trypsin inhibition is a reversible reaction involving thiol-disulphide exchange; that the inhibited complex can be reactivated by oxidants; but that high concentrations of oxidants result in irreversible cleavage of the significant disulphide bond in trypsin, with permanent loss of activity. The inhibitory effect of glutathione on trypsin and protection by glutathione of trypsin-digestible substrates has been confirmed by others [27, 104]. Should any trypsin survive this cytosolic shield and escape into the pancreatic interstitium, it would encounter powerful inhibitors such as alpha-1 antitrypsin and beta-2 microglobulin.

Glutathione that is diverted for trypsin control in hereditary pancreatitis kindreds would compromise its availability for other vital functions: signal transduction; maintenance of redox balance; control of reactive oxygen species that are generated for many physiological roles; removal of reactive xenobiotic metabolites; cysteine replenishment; and protection of trans-sulphuration enzymes that ensure methyl flow (Figure 1) [27, 32]. The methyl-thiol shortfall rationalizes pancreatitis attacks. It also explains electrophilic stress as in studies from Cleveland [105] and Nantes [106]. Both found elevated superoxide dismutase in erythrocytes of patients as well as unaffected family members. Erythrocyte glutathione peroxidase was depressed to the same extent in each subset in the first study, but in the second unaffected members had increased levels such that only in patients was the enzyme ratio elevated - indicating unmitigated electrophilic stress. Patients had low selenium and in the Cleveland study also low vitamin E but elevated glutathione transferase. Thus as might have been anticipated, micronutrient therapy ameliorated symptoms in children with the disease (Table 1) [14].

Mutation in the Cystic Fibrosis Transmembrane Conductance Regulator Gene (CFTR)

The pancreatic lesion in cystic fibrosis is a diffuse form of chronic pancreatitis, the inflammatory stigmata lost when acinar tissue atrophies completely [107]. Hence observations on the pathogenesis of cystic fibrosis should be relevant to chronic pancreatitis, and *vice versa*. In 1998 two groups reported an increased frequency of *CFTR* mutations in patients with chronic pancreatitis [108, 109] and some with recurrent acute pancreatitis [109]. A recent review concluded that idiopathic chronic pancreatitis may occur with one abnormal allele but possession of two (as is usual and in some may represent a *forme fruste* of the disease [16]) confers a 40-fold increase in risk, rising to 500-fold when a *SPINK1* mutation is present too [110]. Why?

The high concentration of trypsinogen in serum of the cystic fibrosis neonate falls exponentially in line with loss of acini until all are eradicated within the first decade. The pattern suggests a permanent blockade to apical exocytosis [15, 17, 111, 112], whereas that is an isolated event in acute pancreatitis (as after ERCP discussed above), but returns sporadically in recurrent acute pancreatitis and with greater frequency and duration in chronic pancreatitis [15]. Thus *CFTR* mutations could facilitate chronic pancreatitis by hindering exocytosis [15, 17, 112]. This interpretation requires the presence of CFTR in the luminal membrane of the normal acinar cell, as is indeed the case [113]. Studies using fluorescent probes, or confluent/semi-confluent cells that retain their secretory polarity [114] or, better still, studies on the isolated perfused cystic fibrosis pancreas, are needed to decide the concept's validity in relation to acinar cells: using the first approach we have shown a failure of antibiotic exocytosis in upper airway cells [115]. The distinction between apical and basolateral discharge is

difficult to make using secretagogues on acinar suspensions [112] because basolateral channels are sensitive to regulation too [19].

The findings of hyper-trypsinogenaemia in neonate cystic fibrosis carriers [116], and of the increased severity of caerulein pancreatitis in *CFTR* heterozygote mice [117] (but with redundant ductal channels for chloride transport), again point to the need for a full complement of CFTR protein for apical exocytosis. They do more, suggesting that the CFTR deficit is exposed by electrophilic strain, in that neonatal antioxidant systems are precarious [118], and, as noted earlier, experimental acute pancreatitis is detonated by an electrophilic burst. With evidence for continuous CYP-mediated strain in chronic pancreatitis and symptom control by micronutrient therapy irrespective of *CFTR* status [108], the obvious next question was whether CFTR may be targeted by toxic electrophiles [15, 27]. Recent studies confirm that it is [119, 120]. Oxidants as in cigarette smoke decrease *CFTR* expression and compromise CFTR function *in vivo* (as gauged by nasal potential difference studies) and *in vitro* [119]. Conversely, ascorbic acid promotes channel opening [121] as also do thiols [122].

It is no surprise that the 90% reduction in CFTR protein in compound heterozygotes with idiopathic chronic pancreatitis results in abnormal nasal potential difference and sweat tests [110]. The key point is that in these sites high levels of CYP expression persist into adulthood [123], such that an increase in toxic electrophiles would impair CFTR function in the absence of *CFTR* mutations. Abnormal sweat tests in the following disparate groups can now be rationalized:

i)   African patients with alcoholic chronic pancreatitis [124];

ii)   Indian patients with trisomy 21 [125] - wherein an extra copy of superoxide dismutase increases the yield of reactive oxygen species; and

iii) patients with kwashiorkor-marasmus [126] who have an absolute lack of defence to elctrophiles [31].

Germane to these arguments, both abnormal sweat tests and elevated serum trypsinogen are documented in malnourished Canadian children [127, 128]. Of note too, nasal potential difference studies indicate CFTR dysfunction in patients with recurrent acute pancreatitis [129,130], as is associated with pancreas divisum [129], in keeping with electrophilic strain [5].

The final piece in the jigsaw of chronic pancreatitis pathogenesis, namely, intraductal calcifying precipitates in many patients with large-duct disease, is provided by new evidence that CFTR in the luminal membrane of duct cells is a channel not only for chloride but also for bicarbonate [131, 132] and glutathione [133]. A reduced quota of CFTR in chronic pancreatitis ductal cells (whether due to *CFTR* mutation, toxic metabolic stress via CYP or other means) would result in less bicarbonate and glutathione concentrations in pancreatic juice at a time when

(between attacks) protein secretion from acinar cells is excessive and/or abnormal [4, 15, 27].

Experimental studies show that progressive acidification of acinar and ductal lumena jeopardizes endocytosis of shed granule membranes and impairs the solubilization of secreted (pro)enzymes, leading to the histological picture of both cystic fibrosis and large-duct chronic pancreatitis [134]: indeed, the importance of bicarbonate is underlined by a study of mice with disrupted cilia function [135]. The way in which bicarbonate lack could compromise removal of the calcium shield from secreted mucus to facilitate protein plug formation has been described [131], as also the key role of glutathione in lysing disulphide bonds in mucus [133]. In our unpublished preliminary work, concentrations of glutathione in pure pancreatic juice (collected endoscopically in the first 10 minutes after secretin) were 1.11, 1.67, 2.02 and 3.02 μmol/L in four healthy controls, but 0.29 μmol/L in a patient with idiopathic calcific chronic pancreatitis, increasing to 2.50 μmol/L after eight months on micronutrient therapy.

Miscellaneous

i)   Mutation in the lipoprotein lipase gene predisposes to recurrent pancreatitis: micronutrient therapy controlled attacks without lowering serum triglycerides in three Manchester patients, of whom a young woman with small-duct chronic pancreatitis had undergone multiple pancreatic operations culminating in a failed attempt at total resection [136]. In a study from Taiwan, *CFTR* mutation rate was 26% in the group with hypertriglyceridaemia and pancreatitis, compared to 1.3% in the group without pancreatitis [137].

ii)   Primary hyperparathyroidism is linked to chronic pancreatitis: in a study of 826 patients, only the subset with a history of pancreatitis had a mutation in *SPINK1* and/or *CFTR* [138].

iii)   Primary haemochromatosis usually causes pancreatic fibrosis, not classical chronic pancreatitis: intravenous micronutrient therapy offered rapid relief in an emaciated woman with calcific disease and a mass in the head of the gland, overcoming by thiols the potential danger of giving vitamin C in the presence of free iron [139].

A New Template for the Pathogenesis of Chronic Pancreatitis

This can be constructed (Figure 2) to accommodate all the information discussed so far. It now becomes possible to see that when environmental and genetic factors combine to cause methyl-thiol lack, as in tropical chronic pancreatitis, the disease begins at a young age and runs an accelerated course. Permutations and combinations among CYP induction, trypsin-favouring mutations, diet, and ductal CFTR involvement would determine outcome - whether large or small-duct chronic pancreatitis, or recurrent acute pancreatitis.
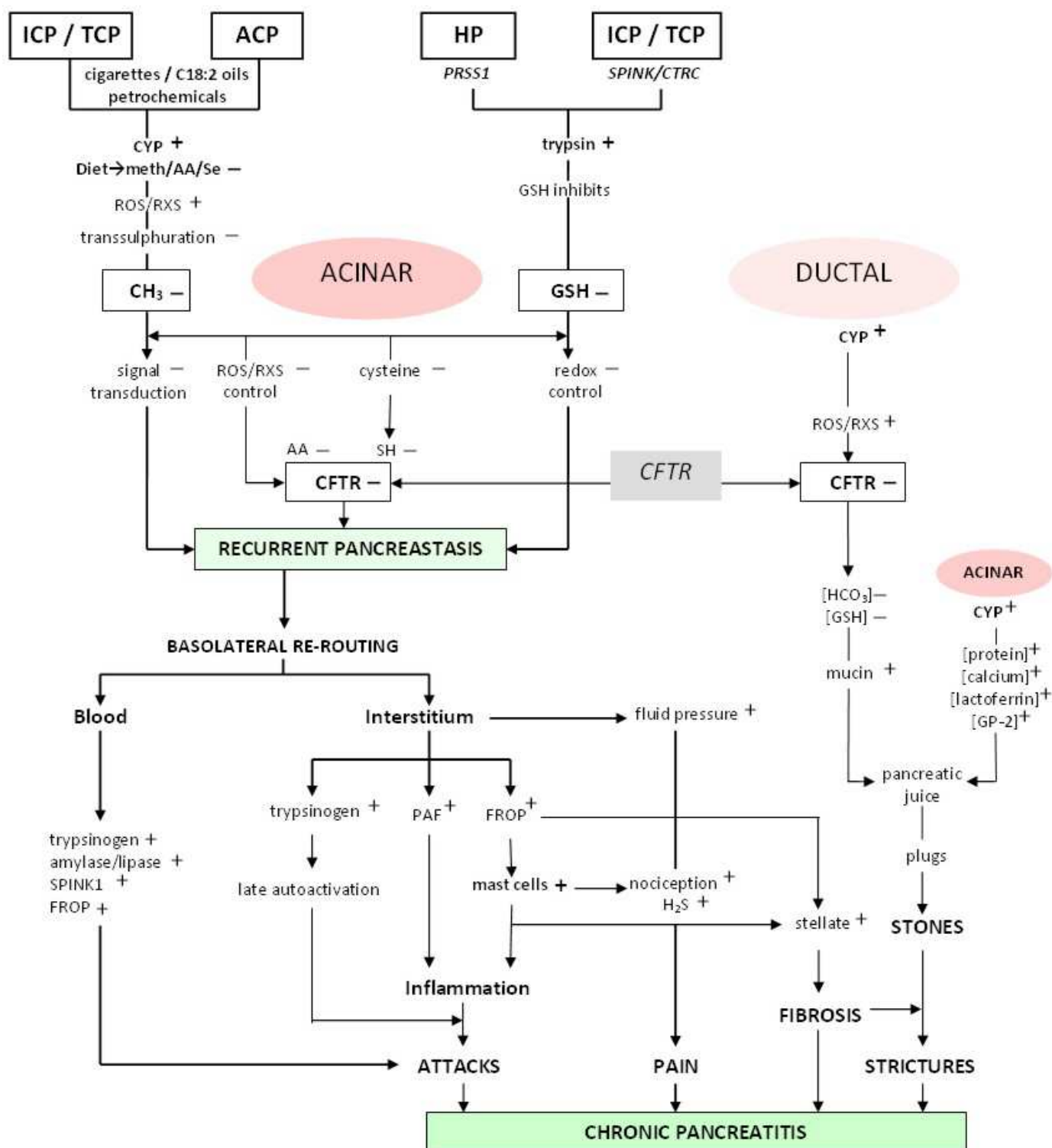
Figure 2. A template for the pathogenesis of chronic pancreatitis based upon arguments in the text. Environmental (top 2 blocks on left) and genetic (top 2 on right) factors come together via lowered CH_3 (methyl)-GSH (glutathione, main thiol) status in acinar cells to cause a secretory block, 'pancreastasis' [17]. A fall in functional CFTR (cystic fibrosis transmembrane conductance regulator protein) compounds that threat, while its loss in duct cells drives towards large-duct disease. Arrows indicate consequences of preceding events. Note the bidirectional relationship between methyl and thiol status wherein methyl lack signifies lack of sulph-adenosylmethionine for GSH synthesis by way of cysteine; while low GSH leaves trans-sulphuration enzymes vulnerable to attack by ROS/RXS and thereby jeopardizes delivery of methyl groups for signal transduction. Plus symbols represent activation/increase, negative symbols decrease/inhibition. Gene mutations are shown in italics. Abbreviations are listed alphabetically. AA: ascorbic acid; ACP: alcoholic chronic pancreatitis; C18:2: linoleic acid; CFTR: cystic fibrosis transmembrane conductance regulator protein; CYP: cytochromes P450; HP: hereditary chronic pancreatitis; FROP: free radical oxidation products; GP-2: protein of the zymogen granule membrane analogous to that in renal casts; GSH: glutathione; H_2S: hydrogen sulphide; HCO_3^-: bicarbonate; ICP: idiopathic chronic pancreatitis; meth: methionine; PAF: platelet activating factor initially from the acinar cell and then the mast cell [17]; ROS: reactive oxygen species; RXS: reactive xenobiotic species; Se: selenium; SH: non-protein thiols; TCP: tropical chronic pancreatitis.

Corollary 2: Understanding Demography

There is a need to explain:

i) the high frequency of chronic pancreatitis in underprivileged communities [33, 140];

ii) 40% concurrence of gallstones and chronic pancreatitis in China where alcoholic chronic pancreatitis is infrequent [141];

iii) susceptibility of African Americans to both chronic pancreatitis and pancreatic cancer [3, 142]; and

iv) increased risk of that tumour in patients with chronic pancreatitis [3, 15, 16].

All this is rationalized within the framework of CYP-related pathology, with poor pre-morbid intake of methyl/thiol precursors as a linking thread. The validity of the explanation is underlined, respectively, by:

i) low micronutrient status from foodstuffs inaffordability, not increased oxidation due to 'Bantu siderosis' [143], in non-alcoholic and alcoholic controls at Soweto where alcoholic chronic pancreatitis is rife [33], as also from hostile cooking practices in Madras where idiopathic disease predominates[75];

ii) dietary/ biochemical studies in relation to gallstones [144, 145];

iii) studies showing high intrinsic CYPA1 activity in African Americans [146], which would amplify the risk from reactive xenobiotic metabolites;

iv) high intrinsic activity of CYP2A6 which tends to bioactivate procarcinogens [147], the roles of reactive oxygen and xenobiotic species in carcinogenesis in general [148] and, in particular, the link between low methyl status and pancreatic cancer [55, 149].

Corollary 3: Opportunity for Prophylaxis

There is an opportunity for prophylaxis by a daily micronutrient tablet in groups at high risk of chronic pancreatitis, as in hereditary pancreatitis kindreds, patients lacking lipoprotein lipase and workers in particular industries. Population prophylaxis should be considered in areas where chronic pancreatitis is endemic. The scientific way would be to tailor tablet composition according to identified need: vitamin C with beta carotene in south India [75], or with selenium in Soweto [33]. The practical solution may be a daily tablet of a compound formulation. Apart from humane considerations, the economic gain should be considerable.

Conclusion

Hitherto environmental and genetic "causes" of chronic pancreatitis seemed to be poles apart. The success of micronutrient therapy across the board makes it possible to draw together these two pathogenetic strands. Chronic pancreatitis emerges as a good (perhaps the best) example of "chaos theory" as applied to medicine: an electrophilic strike on a metabolic pathway that has devastating consequences. Fortunately in this instance there is a simple way of prevention.

Conflict of interest None

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