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A systems biology approach to genetic studies of pancreatitis and other complex diseases

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Abstract. Pancreatitis is usually inflammation of the pancreas without infection. Our understanding of pancreatitis has been built on autopsy studies, surgical biopsies and surrogate markers of inflammation and fibroses, including abdominal imaging techniques and pancreatic functional studies. However, the discovery that a number of different environmental factors and various genetic abnormalities are seen in patients with similar appearing pancreatitis phenotypes teaches us that end-stage pathology is not the disorder. Understanding complex associations and interactions requires that the components and their interactions be organized, stratified and functionally defined. Systems biology, in the broad sense, provides the approach and tools to define the complex mechanisms driving pathology. As the mathematics behind these pathways and mechanisms are defined and calibrated, the potential pathology of patients with early signs of disease can be predicted, and a number of patient-specific targets for intervention can be defined.

Keywords. Pancreas, pancreatitis, acute pancreatitis, chronic pancreatitis, fibrosis, stellate cell, inflammation, systems biology, genetics, genetic testing, meta-analysis, cystic fibrosis transmembrane conductance regulation, trypsin, trypsinogen, pancreatic secretory trypsin inhibitor, CFTR, PRSS1, SPINK1, SAPE, alcohol, tobacco, smoking.

Introduction

Use of systems biology in the study of pancreatic diseases is in its infancy. Mathematical modeling has been used in attempts to understand the electrophysiology of bicarbonate secretion [1, 2] and to predict the effects of functional variations in the cystic fibrosis transmembrane conductance regulator (CFTR) [2]. But it has not been used to study organ-level regulation and dysfunction in a complex medical condition such as pancreatitis. In this review we will describe the major pancreatic inflammatory diseases, review mathematical models of bicarbonate secretion, and discuss approaches and limitations to applying mathematical modeling and analytic tools to the direction and design of future research in pancreatitis.

Normal pancreas structure and function

The pancreas is a gland which is located behind the stomach and that can be divided into three parts, with three functions, facilitated by three types of cells. These three components are united by embryological origin, structural organization, and functional interdependence. The pancreas is an endocrine gland that secretes insulin and other regulatory peptide hormones that arise from endocrine cells in the islets of Langerhans. The pancreas also serves two interacting exocrine functions related to digestion of nutrients within the small intestine. The acinar cells synthesize the pancreatic digestive enzymes, which must be transported to the duodenum where they are activated to digest nutrients that are passing out of the stomach. The duct cells form the pathway linking the acinar

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cells to the intestine through a branching duct system, and produce a bicarbonate-rich fluid that keeps the activating enzymes from the acinar cell in an inactive state, and flush the enzymes out of the duct and into the intestine. The sodium bicarbonate-rich fluid from the duct cells serves to neutralize the hydrochloric acid in the chyme coming from the stomach. A chemical reaction forms sodium chloride and carbon dioxide, and brings the chyme to the neutral pH required for optimal activity of the pancreatic and intestinal digestive enzymes. The entire system is controlled by neurohormonal feedback mechanisms with integration of all sensory information in the dorsal vagal complex of the medulla oblongata, and function is driven by vagal efferents that extend to intrapancreatic ganglion, and on to the duct cells, the acinar cells, and the islet cells [3, 4]. The acinar cells are dependent on the islet cells for proper function as they are bathed in the blood draining from the islets (islet-acinar axis [5]), and the connection of the acinar cells to the intestine is dependent on the ducts – formed by the duct cells.

Historical approaches to inflammatory disorders of the pancreas

Pancreatic diseases

The pancreas is protected from most diseases because of its location and function. The pancreas lies in the retroperitoneal space behind the stomach and intestines, and is shielded from frontal injury by the rib cage and visceral organs (including the liver on the right side and spleen on the left side) and from the back by the spinal column, ribs and muscles. The pancreas does not come into direct contact with the environment as do the skin, lungs and gastrointestinal tract, and does not filter or metabolize most metabolic toxins or xenobiotics. The blood supply to the pancreas is also favored because any toxins, bacterial products or other agents coming from the gut are first filtered by the liver, and then by the lung before reaching the pancreas. Finally, there are only a few infectious agents (e.g. mycoplasma, coxsackievirus, mumps virus, *Ascaris lumbricoides* [6], see Table 1) that occasionally target the pancreas. The major environmental factors that are known to affect the pancreas (e.g. excessive alcohol consumption and cigarette smoking) are factors that can be quantified more accurately than most other exposures using epidemiological techniques. Taken together, these factors make pancreatic diseases less common than diseases in organs directly exposed to injurious agents, and also make the pancreas a useful model system in which to study complex disorders.

Diseases of the pancreas are generally divided into endocrine disorders (e.g. diabetes mellitus), exocrine disorders (e.g. chronic pancreatitis) and neoplastic disorders. Primary endocrine disorders and neoplastic disorders will not be considered here, although they may arise as a complication of exocrine disorders.

Historical classification of exocrine pancreatic diseases

Diseases of the pancreas are not classified according to disease mechanism (see review of classification in [7]). Instead, classification systems followed the pathway of our acquisition of knowledge about the pancreas. Because the pancreas is difficult to access anatomically and is a non-bony structure, the gland remained beyond the reach of the classic physician's tactile abilities and radiological observations. Knowledge of the pancreas's function and diseases were therefore based on animal studies and autopsy studies. Indeed, exocrine diseases of the pancreas were defined by the histologic appearance of inflammation and sclerosis (hardening and thickening of the pancreas associated with scaring and fibrosis) and the clinical triad of malabsorption of food (a very late complication), pancreatic calcifications on abdominal X-rays (a highly variable finding) and diabetes mellitus (a highly nonspecific finding). The tremendous advances in body imaging and functional evaluation of the pancreas developed in the 1970s, 1980s, and 1990s provided additional details to our knowledge about the pancreas's structural and functional demise, allowing us to make an autopsy diagnosis in a living person.

Most diseases of the exocrine pancreas were identified as inflammatory diseases, and subdivided into two non-overlapping categories: acute pancreatitis and chronic pancreatitis [8–10]. Additional classifications were developed that reflected the progressive destruction of the gland; the most widely recognized and utilized the Cambridge Classification [11], which bases disease severity on the progressive distortion of the ductal system as imaged by endoscopic retrograde cholangiopancreatography (ERCP). Currently, most clinicians classify acute pancreatitis as an acute inflammatory condition that last less than 6 months and resolves, while chronic pancreatitis is an inflammatory disorder lasting more than 6 months and may progress to sclerosis [7, 12].

The criteria for the diagnosis of acute pancreatitis and chronic pancreatitis are based on combinations of signs and symptoms that reflect inflammation, anatomical changes and loss of normal function [13–18]. The problem with these diagnostic criteria is that they reflect the pathology, but not the process; the final destination but not the pathway. Such criteria also

Table 1. Etiology of acute pancreatitis (modified from Draganov and Forsmark [6])

Alcohol	Idiopathic
Autoimmune pancreatitis	Infection
Biliary calculous disease	Bacterial
Macrolithiasis (bile duct stone)	Campylobacter jejuni
Microlithiasis (biliary crystals)	Legionella
Biliary cystic disease	Leptospirosis
Choledochal cyst	Mycobacterium avium complex
Choledochocele/duplication cyst	Mycobacterium tuberculosis
Congenital anomaly	Mycoplasma
Annular pancreas	Parasites/worms
Anomalous pancreato-biliary junction	Ascaris lumbricoides
Pancreas divisum	Clonorchis sinensis
Duodenal obstruction	Microsporidia
Afferent limb obstructed (Billroth II)	Viral
Atresia	Coxsackievirus
Crohn's disease	Cytomegalovirus
Diverticulum	Echo virus
Drugs	Epstein-Barr virus
Acetaminophen	Hepatitis (A, B, C) virus
Azathioprine	HIV
Didanosine	Mumps virus
Erythromycin	Rubella virus
Estrogen	Varicella virus
Furosemide	Metabolic
Histamine-2 receptor antagonists	Hypercalcemia
Mercaptopurine	Hyperlipidemia
Methyldopa	Renal disease
Metronidazole	Chronic renal failure
Nitrofurantoin	Dialysis related
Nonsteroidal anti-inflammatory agents	Sphincter of Oddi dysfunction
Pentamidine	Toxin
Tetracycline	Organophosphate insecticides
Valproic acid	Scorpion bite
Iatrogenic	Trauma
ERCP	Tropical
Abdominal surgery	Vasculitis

describe general disease associations and not mechanisms.

From an environmental risk perspective, the etiology of acute and chronic pancreatitis is usually presented as a long list of factors that have a statistical association with the disease in a cases-series or an epidemiological study [6, 19–22] (See Tables 1 and 2). Excessive alcohol use, for example, is one of the most common causes of chronic pancreatitis [23], but most individuals who drink excessive alcohol do not develop pancreatitis [24]. Furthermore, in a large proportion of patients diagnosed with chronic pancreatitis, no single factor can be found, including family history [7]! Finally, insights into the various etiologies of acute and chronic pancreatitis are not gained by the appearance of the pancreas on imaging studies, and even histology studies, since they are all similar, regardless of etiology [25].

Animal models are equally unhelpful. Numerous animal models of pancreatic inflammation have been developed [26], but their relevance to human disease remains uncertain, since it was unclear what

Table 2. Etiology of chronic pancreatitis (Modified from TIGAR-O Version 1 [59]).

Toxic-metabolic
Alcoholic
Tobacco smoking
Hypercalcemia
Hyperparathyroidism
Hyperlipidemia (rare and controversial)
Chronic renal failure
Medications
Phenacetin abuse (possibly from chronic renal insufficiency)
Toxins
Organotin compounds (e.g., DBTC)
Idiopathic
Early onset
Late onset
Tropical
Tropical calcific pancreatitis
Fibrocalculous pancreatic diabetes
Other
Genetic
Autosomal dominant
Cationic trypsinogen (codon 29 and 122 mutations)
Autosomal recessive/complex
CFTR mutations
SPINK1 mutations
Autoimmune
Isolated autoimmune chronic pancreatitis
Syndromic autoimmune chronic pancreatitis
Sjögren syndrome-associated chronic pancreatitis
Inflammatory bowel disease-associated chronic pancreatitis
Primary biliary cirrhosis-associated chronic pancreatitis
Recurrent and severe acute pancreatitis
Postnecrotic (severe acute pancreatitis)
Recurrent acute pancreatitis
Vascular diseases/ischemic
Postirradiation
Obstructive
Pancreatic divisum
Sphincter of Oddi disorders (controversial)
Duct obstruction (e.g., tumor)
Preampullary duodenal wall cysts
Posttraumatic pancreatic duct scars

was being modeled. The concern is further heightened because treatments that are effective in animal models are ineffective in human disease [27]. Thus, progress in this field remained in a standstill.
End of *status quo*.

Arguments for a paradigm shift in approaching pancreatic diseases

Mechanistic definitions of acute and chronic pancreatitis

The definitions of acute pancreatitis and chronic pancreatitis listed above are totally inadequate from a mechanistic perspective [7]. In understanding a disease process, we want to know why a person develops inflammation, why it affects the pancreas, why the amount of inflammation varies among patients, why the duration of inflammation varies, why the manifestations of inflammation vary, why the complications vary and why some people recover while others progress. Furthermore, we need to be able to predict these variables in individual patients so that optimal, patient-specific interventions are developed and deployed to prevent development of conditions that cannot be reversed. Thus, our group has advocated changes in the definitions of acute and chronic pancreatitis that are more mechanistic and reflect observed pathophysiology. We define acute pancreatitis as an acute injury to the pancreas resulting in an acute inflammatory response. We also define chronic pancreatitis as chronic inflammation of the exocrine pancreas (regardless of the type or severity of dysfunction). Thus, acute pancreatitis is an *event*, while chronic pancreatitis is a *process*. The clinical signs and symptoms of the various syndromes associated with specific processes and complications of acute and chronic pancreatitis can then be described.

Requirements for a paradigm shift in pancreatitis models

The discussion above serves as a microcosm of the challenges in understanding and treating chronic disorders. We believe that three fundamental problems exist in the current approach to clinical research that impede consistent and rational progress in discovery. First, classification of medical disorders is historically based on autopsy studies. Second, the methods of discovering mechanisms of disease have relied on variations of case-control and statistical test design, and are reductionist rather than integrative. Third, animal models of complex diseases of unknown etiology have been a focus of study. However, treatment studies based on the average (i.e. normal) responses of model animals and healthy humans have failed to provide useful treatments for inflammatory pancreatic diseases, suggesting that there could be fundamental flaws in older paradigms used to understand pancreatic disease. Furthermore, the older paradigms are linked with methodological approaches that are limited, and must also be reconsidered.

Progress will require a paradigm shift in these three areas, recognizing the following fundamentals. First, complex disorders result from the failure of *abnormally functioning pathways*. In other words, complex disorders result from pathologic failures of metabolic or homeostatic systems because they are intrinsically flawed at multiple levels, steps, or pathways, rather than exposure to overwhelming external or internal insults. Second, complex disorders can encompass multiple steps and multiple pathways that differ between individuals and populations, but can still result in one pathology-based phenotype. Thus, the likelihood of understanding the effect of a single variable in a complex, highly variable context using the case-control method of normal subjects and end-stage disease is very limited. Using meta-analysis to combine data for multiple studies from multiple populations to understand complex disorders based on end-stage pathology actually gives less information, rather than more [28, 29]. Third, in complex human disorders, *all* of the subjects with a disorder have multiple disease-associated genetic factor exposures or metabolic conditions linking non-disease to disease. However, *most* of the controls, who by definition do not have the specific pancreatic disorders, will also have a variety of disease-associated genetic variables, environmental exposures and metabolic conditions, but the *combination* of risk factors in these persons does not lead to inflammatory diseases of the *pancreas*. If a complex disorder reflects a variety of abnormal pathways, then it should not be surprising that a single treatment approach, designed in normal animals and targeting a single factor usually fails to be of significant benefit in the majority of individuals within a population of subjects with complex disorders.

Definition of systems biology for inflammatory pancreatic disease

Systems biology has been defined in a number of ways. In the current context we would view systems biology as the study of the integrated interaction of genes, proteins, pathways, cells and organs that allows for homeostatic structure-function relationships to preserve and perpetuate a living organism in a hostile environment. Since systems approaches address a complex disease in its entirety, as opposed to reductionist approaches that examine mechanisms in isolation, it may prove to be of both basic and clinical utility [30]. Such an approach to discovery makes extensive use of interpretative models that are iteratively improved through the acquisition of empirical data from targeted experiments suggested by such models. Thus, improved understanding of pancreatic diseases will ensue from improved understanding of the function and dysfunction of the various components, and be able to integrate, through mechanistic models, the various dysfunctional components in a systematic way. From this perspective we can approach every aspect of pancreatic disease from a highly mechanistic perspective and couch hypotheses and experiments accordingly.

Elements of complex pancreatic diseases

The key factor in non-infectious inflammatory diseases of the pancreas

We note that the pancreas is well protected from environmental and metabolic insults by its location, its blood supply and its functions. The pancreas, however, behaves differently than other glands because it elicits an inflammatory response that is out of proportion to inflammation caused by an equivalent insult in other organs. Although the theory of inflammation associated with pancreatic digestive enzyme activation and pancreatic autodigestion was first proposed in 1896 by Chiari [31], the critical proof did not come for 100 years until the discovery in 1996 that cationic trypsinogen gene (*PRSS1*) mutations caused hereditary pancreatitis [32]. Since then a number of trypsinogen gene mutations have been discovered that are associated with both acute and chronic pancreatitis through alterations that cause premature and sustained trypsin activity inside the pancreas [33, 34]. Although most patients with acute pancreatitis or chronic pancreatitis do not have trypsinogen mutations, the functions and dysregulation of trypsin appear to be the primary differences between inflammatory disorders of the pancreas and disorders in other exocrine and endocrine glands.

The inappropriate activity of trypsin may be the Achilles' heel of the pancreas. Trypsin is very dangerous because (a) it can cause direct injury to tissues by digesting proteins, (b) it activates other digestive zymogens and (c) it cross-activates the immune system. Thus, the critical protective mechanisms for the pancreas are directed at protection from trypsinogen activation and prevention of sustained trypsin activity. The mechanisms of premature trypsinogen activation and the locations of injury can be divided into three primary domains: the acinar cell, the duct and the interstitial spaces.

Trypsinogen activation and trypsin survival are regulated by calcium [35]. It appears that the primary mechanism to prevent trypsin injury inside the acinar cell is to maintain calcium at low levels [36]. Processes that elevate intracellular calcium levels in acinar cells, including fatty acid-mediated ATP depletion [37] and

apical bile salt exposure [38], will lead to trypsinogen activation and pancreatitis [39].

Once trypsinogen is secreted into the duct, the calcium-dependent mechanisms utilized by the acinar cell for protection from trypsin become irrelevant because the calcium levels in the duct are quite high. Instead, the duct is protected through maintenance of an alkaline pH and by rapid flushing of the zymogens and prematurely activated enzymes out of the pancreas and into the duodenum. Duct physiology is dependent on normal CFTR function, and failure of the duct because of *CFTR* mutations [2] or distal ductal obstruction limiting pancreatic juice flow will predispose to unregulated intrapancreatic trypsin and acute pancreatitis. Indeed, gallstones or sludge that intermittently obstruct the pancreatic duct and disrupt this protective mechanism appear to be the most common cause of acute pancreatitis [40–42].

The impact of trypsin activity in the interstitial space has not been fully elucidated. However, there are a number of reasons to suspect that it may be important. But this speculation is beyond the scope of this review.

Major genetic factors in acute and chronic pancreatitis.

Genetic factors are now recognized for their important role in acute pancreatitis. The key genetic factors that increase susceptibility to acute pancreatitis all disrupt the protective mechanism limiting trypsinogen activation and trypsin inactivation [35]. In the presence of these genetic effects the threshold for triggering unregulated intrapancreatic trypsin activation is lowered, and smaller environmental insults or metabolic stressors will initiate injury.

The prototype gene-associated DNA sequence variants are the cationic trypsinogen gene (*PRSS1*) and the cystic fibrosis transmembrane conductance regulator gene (*CFTR*) [35, 43]. The pancreatic secretory trypsin inhibitor (PSTI) is an acute phase reaction protein which is unregulated *after* pancreatic injury and in the context of inflammation [44]. Mutations in the PSTI gene (*SPINK1*) are associated with idiopathic pancreatitis in children [45, 46], tropical pancreatitis [47–50], familial pancreatitis [46] and other types of pancreatitis [51, 52]. These three genetic factors are all strongly associated with susceptibility to pancreatitis through trypsin-associated injury, either by altering trypsin's calcium-dependent molecular regulatory mechanisms (*PRSS1* mutations), diminishing the pancreatic duct's pH regulating and flushing capacity (*CFTR* mutations) or failing to inhibit sustained trypsin activity after injury is initiated (*SPINK1* mutations).

Surprisingly, most individuals with these pancreatitis-associated mutations rarely develop attacks of acute pancreatitis, and only a subset with recurrent acute pancreatitis develop chronic pancreatitis [44, 53]. Furthermore, mutations in the susceptibility genes do not determine the severity of injury or the types of complications. The susceptibility factors do not directly interact with the environmental agents that appear to be the cause of acute pancreatitis or chronic pancreatitis. Alcohol consumption and tobacco smoking, which appear to be among the strongest risks factors for developing acute and chronic pancreatitis, are examples of an apparent mechanistic disconnection between the environmental factors and genetic factors. Thus, the mechanisms are multi-dimentional and very complex.

The etiology of acute pancreatitis also appears to be disconnected from disease severity. For unclear reasons, only about 20 % of patients with acute pancreatitis develop a severe clinical course with the systemic inflammatory response syndrome (SIRS) [42]. Now some genetic [54, 55] and metabolic factors [56, 57] are being recognized as playing an important role, but they are all downstream of the susceptibility factors. In chronic pancreatitis, susceptibility factors are also disconnected from the amount of fibrosis, the severity of pain, the presence of calcifications, the development of diabetes or risk of early cancers [58]. Thus, in studying patients with pancreatic inflammation, the disease onset, character, severity and complications remain unpredictable. Understanding susceptibility is only the first step.

Defining new goals and developing new approaches

The goal of our current research is to gain a detailed understanding of the biology of each of our patients, to understand the *specific* risk for each patient, and to be able to prescribe a highly targeted and specific treatment for each patient. Our larger goal is to develop individualized medicine that provides a lifetime of health to people who are otherwise doomed to one of the most painful, disabling, frustrating and hopeless group of disorders such as the non-infectious inflammatory disorders of the pancreas. There will be no single answer to the etiology and treatment of all inflammatory diseases of the pancreas because they are complex, multi-step disorders. Therefore, we are compelled to use systematic methods to consider all of the possibilities in each patient. We must determine the locations, connections, interactions and contributions of the major factors, and predict the best site for targeted therapy. This does not yet exist; but it can.

Organizing the sequence of events in inflammatory diseases of the pancreas

Our approach to developing a highly predictive model of the pancreas is to organize all of the factors that contribute to normal pancreatic physiology into mechanistic categories. Second, we are organizing all of the risk factors known to be associated with pancreatic injury, the inflammatory response and all complications [7]. Third, we are organizing possible sequences of events in a pathological process so that each step can be isolated and studied [28, 29]. Fourth, we are identifying biomarkers to precise analysis of each step of the process. In addition, we are designing prospective studies of patients so that each process can be monitored over time. Developing a highly organized structure with quantitative measures at each step allows a complex disorder to be simplified and analyzed with clarity.

The key elements of pancreas anatomy and physiology have been described above. From a modeling perspective we recognize an acinar cell compartment, a duct cell compartment, the stimulatory neurohormonal compartment, the inhibitory neurohormonal compartment, the sensory systems (neural and immune) and the immune system. Within each compartment are functional domains which allow the components to be further organized according to their ability to protect the pancreas from environmental or metabolic stresses, injury, injury sensation, injury containment, injury resolution, and adaptation to recurrent injury [35]. We have also developed hypothetical models of injury and repair that allow the major effectors to be considered in consecutive order, noting that proximal events are necessary for distal events to relevant. Examples of structural models of key molecules, mathematical modes of cell function and dysfunction, pharmacokinetic models of environmental exposure, and influence diagrams of chronic and acute pancreatitis will be presented.

Organizing known processes relevant to chronic pancreatitis

Two fundamental principles were considered in developing a new model of chronic pancreatitis. First, the majority of individuals with major environmental risk factors (e.g. heavy alcohol consumption) do not develop chronic pancreatitis [59]. Second, the destruction of normal pancreatic parenchyma and scarring, which are the hallmark features of chronic pancreatitis, require immune system activation before the disease can progress [60]. In studying histologic sections of chronic pancreatitis, it appeared that macrophages were present in the vicinity of pancreatic stellate cells, which are the cells responsible for the collagen and other matrix proteins of scars and fibrosis [61–63]. We reasoned that the difference between an environmental risk and an environmental etiology was activation of the immune system, which could occur through an episode of acute pancreatitis (pancreatic injury with an inflammatory response). This was termed the Sentinel Acute Pancreatitis Event (SAPE) hypothesis model (Fig. 1) [43, 60, 64]. The advantage of this model was that it allowed the risk factors and roles of the major systems to be organized into sequential phases, with branch points and alternate pathways as downstream events. The timing and upstream requirements for various complications, such as fibrosis or visceral hypersensitivity and pain, could also be organized. In addition, the SAPE hypothesis model allowed for the development of the domain model [35], which organized the functional aspects of the key variables that were all necessary to develop the features of chronic pancreatitis (Fig. 2).

The sentinel event in the SAPE model is an episode of acute pancreatitis. The innate immune system is also a critical executer of the damage in acute pancreatitis [42], but it requires immune activation, usually through acute injury. We recognize that injury can occur in a variety of ways, but the most important injury appears to be related to unregulated trypsin activity. Furthermore, we recognize that trypsin is normally in two different compartments (the acinar cell and the duct), but is released into the interstitial space under abnormal conditions, including secretion directly into the interstitial space during inflammation [65]. Thus, several locations for trypsin activation and injury must be considered. Furthermore, as noted above, a single episode of acute pancreatitis can be life-threatening in up to 20% of individuals [42], probably related to dysregulation of the inflammatory response rather than to the magnitude of injury alone [66]. A model of the events related to a single episode of acute pancreatitis is therefore also complex (Fig. 3).

Connecting acute and chronic pancreatitis

Studies on hereditary pancreatitis linked recurrent acute pancreatitis to chronic pancreatitis and served as a model for the SAPE hypothesis [58, 64]. However, most individual with acute pancreatitis, or recurrent acute pancreatitis, do not develop chronic pancreatitis. How important is the *severity* of the attacks, the *frequency* of attacks or the *duration* of the attacks in predisposing to chronic pancreatitis? All of these variables must be considered, as acute and chronic pancreatitis are linked.

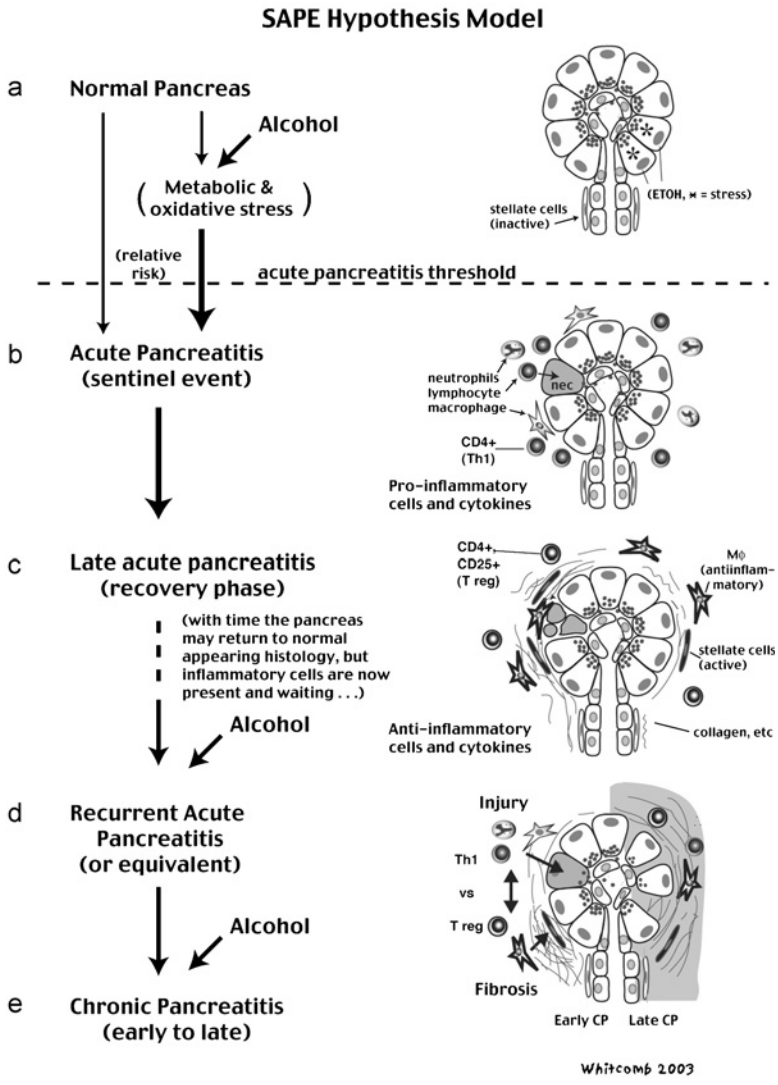

Figure 1. The sequence of events leading to chronic pancreatitis in the SAPE hypothesis model. (*a*) Normal pancreas. If the subject is a heavy alcohol user, the acinar cells are under metabolic and oxidative stress (*), but the histology remains relatively normal. Alcohol increases the risk of crossing the acute pancreatitis threshold (bold line crossing the dashed line). (*b*) Acute pancreatitis with pancreatic injury and infiltration of pro-inflammatory cells. The first, or sentinel Acute Pancreatitis Event (SAPE) is a critical step because it initiates the inflammatory process that results in both injury and later fibrosis. (*c*) Late acute pancreatitis is dominated by anti-inflammatory cells that limit further injury by pro-inflammatory cells and products, and promote healing. This includes activation of stellate cells which produce collagen, etc. In the absence of recurrent acute pancreatitis, acinar cell toxins (e.g. high-dose alcohol) or factors that activate the immune system, the pancreas may eventually return to normal appearing histology – except for some residual inflammatory cells that are primed to respond to any future injury. (*d*) Recurrent acute pancreatitis, acinar cell injury or other factors that activate an acute inflammatory response (Th1) are immediately countered by an anti-inflammatory counter response (T reg) which, among other things, drives fibrosis. This vicious cycle results in both continued injury (top) and further fibrosis (bottom), leading to (*e*) extensive acinar cell loss and sclerosis (right) that is characteristic of chronic pancreatitis. Both genetic factors and environmental factors play a role in this process by increasing susceptibility to acute pancreatitis, altering the severity and duration of acute pancreatitis, and altering the healing processes that drive fibrosis. Alcohol is especially important because it acts at multiple steps in this process. From [64].

Modeling of individual elements within a broad disease model

The elements of a disease model can be viewed from many perspectives. To understand complex human disorders, we needed to consider multiple levels, extending from the functional variants in nucleotides and proteins, to various cell types, to cell communication and integrated organ function. Many of the key proteins known to be involved in the pathophysiology of pancreatic diseases are cell and compartment specific (e.g. CFTR on the duct cells), while others are enzymes, cytokines, and even structural proteins (e.g. collagen) that exist in multiple locations. In understanding human *disease*, the details of the system are of lesser importance if the system consistently functions the same way in all humans. We are more interested in the proteins and cells that are variable, and contribute to a pathologic process.

Medical genetics focuses on DNA sequence variants that alter the expression, regulation or function of protein that are critical to health. The challenge arises because the importance of a DNA variant can only be understood within the context of health and disease. Three examples of modeling are given: (a) modeling the effects of mild *CFTR* mutations that potentially limit bicarbonate, but not chloride conductance; (b) structural studies of trypsin; and (c) pharmacokinetic modeling of alcohol clearance in the presence of mutations in alcohol dehydrogenase gene.

Mathematical modeling of CFTR mutations in pancreatic duct cells

One of the prior mysteries of pancreatic physiology was the mechanism whereby this gland could secrete juice with a bicarbonate concentration approaching 140 mM. We developed a model based on the known channels and transporters within the pancreatic duct

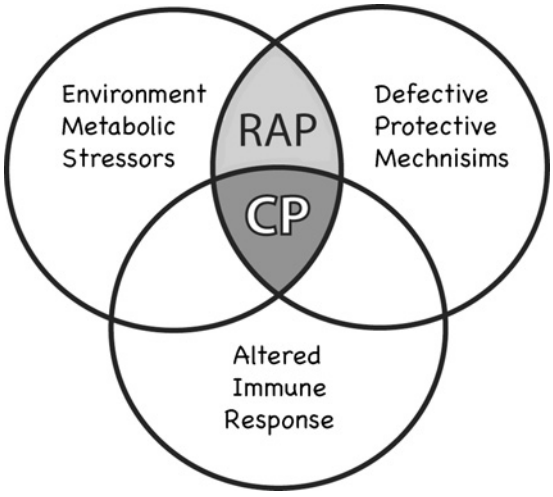

Figure 2. The domain model of chronic pancreatitis. This model focuses on the general interaction of multiple risk factors within three general domains, represented by three overlapping circles. Subjects who have inadequate injury protection (e.g. they have *PRSS1*, *SPINK1* or *CFTR* mutations) and who are challenged by metabolic or environmental stressors (e.g. pancreatic hyperstimulation) develop recurrent acute pancreatitis (RAP). A subset of these subjects seem to have an altered immune response that leads to prolonged episodes of acute pancreatitis (smoldering pancreatitis) or accelerated fibrosis and chronic pancreatitis (CP). Modified from [35].

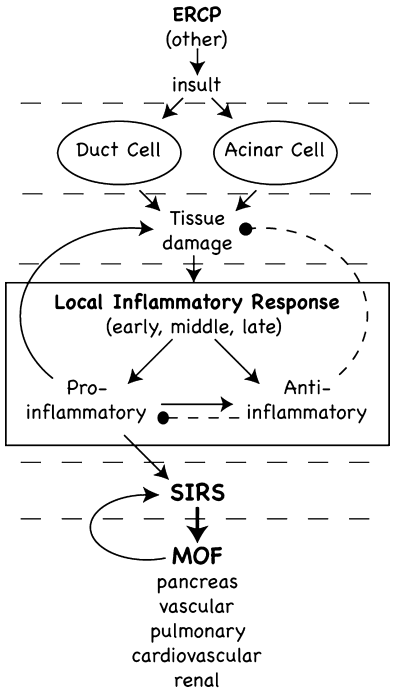

Figure 3. Schematic of the processes in acute pancreatitis. Solid arrows: induction; fine dashed lines: suppression; large dashed lines: thresholds resisting progression. An initiation stimulus (e.g. ERCP) stresses the acinar and/or duct cells leading to tissue damage. Damage stimulates pro- and anti-inflammatory pathways. Pro-inflammatory agents (e.g. TNFα) cause more tissue damage and more inflammation. Anti-inflammatory agents (e.g IL-10, TFB-β1) suppress pro-inflammatory responses and stimulate healing. In some cases the pro-inflammatory response leads to a systemic inflammatory response syndrome (SIRS) and multi-organ failure (MOF). Various organs and systems may be affected.

and proposed a mechanism for high concentration bicarbonate secretion in 1999 [67] (Fig. 4). Since it was technically impossible for us to test this hypothesis in human pancreas, we used mathematical modeling of the pancreatic duct to determine conditions necessary to generate high concentration bicarbonate secretion and test our hypothesis *in silico* [2]. The model was built using previously published parameters for ion channel and exchanger function [1, 68] but arranged under the anatomical constraints of the proximal pancreatic duct. Using this model we were the first to demonstrate the mechanisms used by pancreatic duct cells to generate high concentrations of bicarbonate in pancreatic fluid through a CFTR-dependent mechanism (Fig. 5). In addition, this model allowed prediction of how specific types of *CFTR* mutations would influence the volume and content of pancreatic juice. We predicted that classes of *CFTR* mutations that specifically reduced bicarbonate permeability would have profound effects on pancreatic secretion that mimicked the effects of severe *CFTR* mutations seen in cystic fibrosis. On the other hand, classes of CFTR mutations that only altered chloride permeability, while preserving bicarbonate permeability, had almost no effect on the pancreas. Thus, if bicarbonate-altering polymorphisms were present in a subject, they could not efficiently clear the duct of zymogens and would be susceptible to pancreatitis, while other organs that used epithelial CFTR to transport chloride would be minimally affected [2]. This exercise also demonstrated that mathematical simulation could provide major insights into the pathological mechanisms of complex disorders, and specific insights into susceptibility for pancreatitis initiated inside the pancreatic duct.

Crystallographic studies of trypsinogen mutations

The study of trypsinogen gene mutations has opened the door to the study of inflammatory diseases of the pancreas. Two mutations, *PRSS1* R122H [32] and N29I [69], were the first and most common of the >20 trypsinogen mutations to be associated with hereditary pancreatitis, an autosomal dominant syndrome of recurrent acute and chronic pancreatitis. Studies of the three-dimensional structure demonstrated the physical location of mutant amino acids relative to functional regulation of the molecule [32, 69]. We noted pancreatitis-associated mutations clustered around the calcium binding pockets – which were key regulators of trypsinogen activation and trypsin inactivation – and these mutations appeared to confer

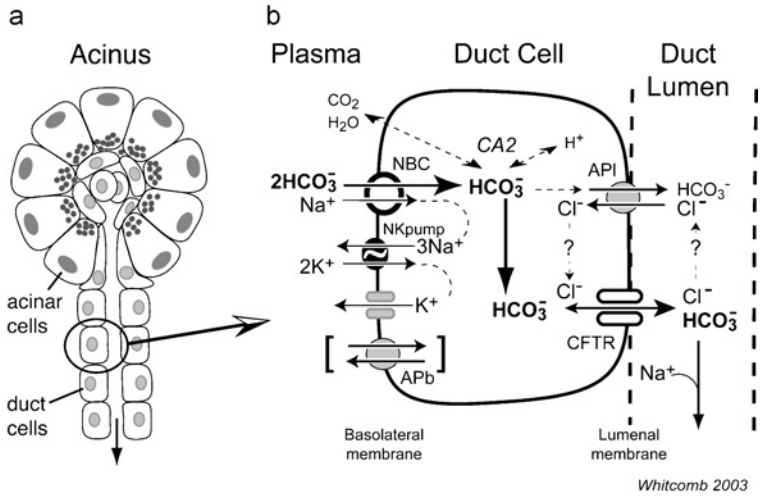

Figure 4. Whitcomb-Ermentrout pancreatic duct cell model. (*a*) Organization of the acinus with the acinar cells forming a cul de sac and with the duct cells, which extend into the acini as centroacinar cells, forming a conduit for the flow of secreted fluid. (*b*) Model of the duct cell used in the complete model. For abbreviations, see text. The symbol AP_b indicates the location of the chloride/bicarbonate antiporter on the basolateral membrane that is inactivated in the standard model. The ? marks the sites of the hypothetical circulation of chloride out of CFTR and in through the AP_l in exchange of bicarbonate during active secretion in previous models that is not active or necessary in the current complete model under standard conditions. From [2].

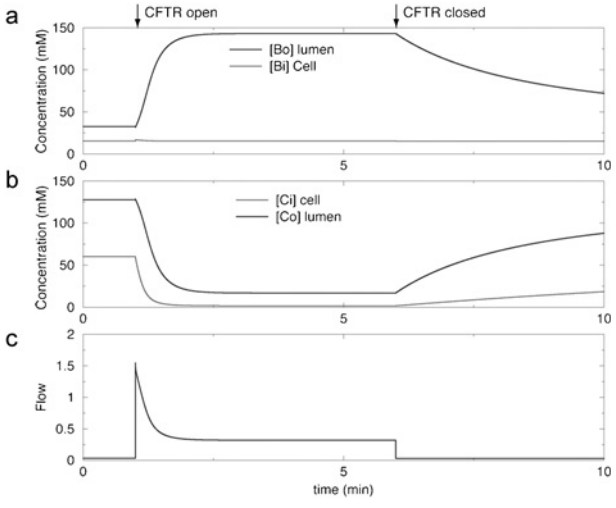

Figure 5. Mathematical modeling of pancreatic secretion. Anion concentrations and flow in the complete duct cell model as a function of CFTR. (*a*) Concentrations of bicarbonate and chloride in the intracellular (i) and luminal (o) compartments as a function of time. Bicarbonate concentrations inside the duct cell (Bi) and in the lumen (Bo). The first arrow marks CFTR opening at 1 min, the second arrow marks CFTR closing after 5 min (i.e. at the 6-min mark). Note that the luminal bicarbonate concentration quickly reaches the target concentration of >140 mM within about 1 min under standard conditions. (*b*) Chloride concentrations plotted as in (*a*) for bicarbonate. Note the relatively low chloride concentrations inside the cell during active secretion. (*c*) Flow of pancreatic juice in arbitrary units. The basal flow is due entirely to secretion of plasma-like fluid from the acinar cells. The fluid volume is closely associated with ion efflux from the duct cell. From [2].

a gain-of-function phenotype [43]. These functional mutations appeared to cause trypsinogen to act as if one of the two calcium binding sites was always occupied, thereby lowering the threshold for inappropriate activation [43]. Since the calcium concentration in the duct is high, these studies, along with laboratory studies on animals and isolated cells [36, 37, 70], provided evidence that acute pancreatitis can be initiated within the acinar cell if calcium levels remain elevated [71]. Thus, modeling of acinar cell calcium and the molecular regulation of trypsinogen provide key insights into susceptibility for pancreatitis initiated within the acinar cell compartment.

Pharmacokinetic modeling of alcoholic pancreatitis with ADH polymorphisms

Alcohol consumption is a common feature in patients with recurrent acute and chronic pancreatitis. Many studies have attempted to link alcoholic pancreatitis with polymorphisms in alcohol metabolizing genes [72–77]. The results have been very disappointing. What is known is that there is a dose-dependent relationship between alcohol consumption and risk of pancreatitis [23], that there are different effects that require a threshold of exposure to change pancreatic physiology [78], and that alcohol exposure decreases the threshold to acinar cell trypsinogen activation [79, 80]. This raises the question of exactly what to expect from polymorphisms in alcohol metabolizing genes.

Sultatos et al. [81] used physiologically based pharmacokinetic modeling linked with Monte Carlo sampling as a tool for the quantification of interindividual variability in chemical disposition and/or response when applied to alcohol metabolism and linked to multiple polymorphisms in the alcohol dehydrogenase (ADH) gene. They demonstrated that the area under the curve for ethanol blood decay was very sensitive to alcohol dehydrogenase genotype, suggesting that these polymorphisms affect peak levels and exposure time.

If susceptibility to alcoholic acute pancreatitis requires a sustained alcohol concentration at the cellular level over a threshold amount, then we would predict that the patients who are most likely to be affected by

ADH polymorphisms are ones who drink a *moderate* amount of alcohol, predicted to be near the threshold level. Compared to normal metabolism, slower alcohol metabolism rates would shift the biological effects of some moderate drinkers over the threshold to a higher risk, while a faster alcohol metabolism would shift the biological effects of others below the high-risk threshold. The polymorphism would only be relevant to moderate alcohol drinkers within two specific windows of doses, just below (slow metabolism) and just above (fast metabolism) the threshold level. Subjects who are light drinkers or heavy drinkers would not be at risk regardless of genotype, and no effect would be seen in slow metabolizers who drink above the window, or rapid metabolizers who drink just below the window of relevant dose. Therefore, the effect of environmental factors that interact with genetic factors must be calibrated within a broader biological mechanism.

We believe that these types of approaches are critical for understanding complex disorders. The reason is that they bring insights into specific steps, and allow interacting processes to be quantified and calibrated as they are applied to experimental data.

Statistical challenges to genetic studies in small, complex patient groups

There are several major challenges to approaching acute and chronic pancreatitis as a classic genetic disease. First, the disease appears to be sporadic, with only hereditary pancreatitis having a clear autosomal inheritance pattern. Second, the environmental risk factors have weak effect size, and there are many possible targets. Third, the diseases are actually syndromes, with a wide variety of features. Fourth, the diseases are classified by end-stage pathology. Fifth, the relevance of animal models is unknown, so insight from animals is limited. Sixth, candidate gene studies have been generally negative, or the effects small and not replicated [82, 83]. Finally, pancreatic diseases are uncommon, so designing a high-powered case-control study (needing many thousands of patients/controls) requires an enormous and very costly effort.

Complex disorders in heterogeneous populations

If chronic pancreatitis, for example, is a complex, multi-step and heterogeneous disorder, then it could be conceptualized within a population, as illustrated in Figure 6. The traditional approach has been to identify all subjects with the pathological signs of chronic pancreatitis (e.g. fibrosis), to find the average signs and symptoms, and to rank the environmental factors in order of prevalence compared to the general population. Although this provides some insight into pancreatitis in general (e.g. Table 2), it offers little predictive value for individuals with exposure to these same environmental factors, and offers little hope for specific preventative strategies. How can the elements be resolved?

Limitations of meta-analysis

Currently, many experts consider the results of meta-analysis, which compares hundreds of patients from multiple genetic studies, to be the strongest evidence of a genetic effect on disease [82–84]. Based on this approach, the general consensus among many leaders in the field is that (a) most genetic susceptibility factors will only have a small effect on common diseases, and (b) studies are generally unreliable unless they have greater than 1000 subjects per study arm [28].

We recently addressed the issues of study size and study design in simple and complex genetic traits and suggested that the problem of non-reproducibility is not study size, but study design [29]. From our perspective, the use of meta-analysis in evaluating multiple studies of very complex disorders (some with possible systematic design flaws) may have been generating erroneous inferences regarding the lack of genetic effect in some common diseases [29]. If the goal of investigating complex traits is to provide insight into disease mechanisms for individual patients, then complex genetics should be approached from what we are calling a *systems-based case series* approach in which multiple genetic effects are considered in independent, and properly powered, mechanistically based studies before the independent effects of the variables are integrated into a complex disease model [28]. The outcome of these studies should be a mathematically based tool that has outstanding performance characteristics on a patient-by-patient basis.

Consider Figure 6. If a single factor were selected (e.g. 3C), the effect of any variation in effect would be very difficult to measure using a case-control design, e.g. the relative frequencies of factor 3C polymorphism in subjects with versus subjects without pancreas fibrosis. The number of subjects needed to determine a significant effect, based on power calculations, would be very large because of the multiple uncontrolled variables that also contribute to fibrosis. A major limitation in using meta-analysis for these studies is that different populations are combined without knowledge of other variables. For example, consider the reproducibility of studies on factor 3C in a population that was only exposed to environmental factor 1D versus a population that was exposed to

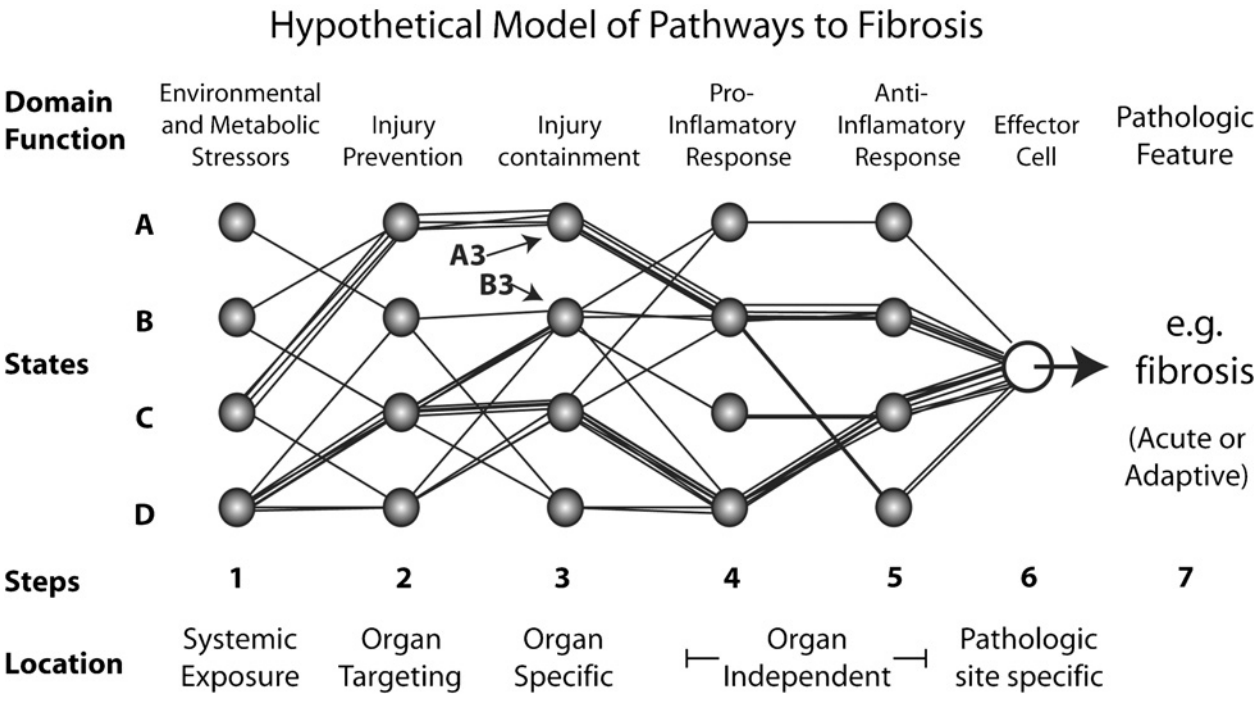

Figure 6. Summary of common pathways chronic pancreatitis. Mapping of pathways of a complex disorder from a normal organ to pathological features (in this case fibrosis) on individuals within a population. The illustration outlines six steps that are sequentially linked from an environmental or metabolic exposure (step 1) to a pathologic feature (e.g. fibrosis), which can be directly pathological, or drive an adaptive response that contributes to the pathological features. Different states (A–D) regulated by unobserved variables are illustrated with a circle. Each line represents a subject with pathology, with the line forming a continuous link between the stressor state (1A–D) and the effector cell (6). Each state represents an agent (e.g. a gene, protein, cell, or organ) that responds to an input with variable sensitivity, and transmits an output with variable effect size, and which is either normal, amplified or diminished in either amplitude, duration, or character, compared to normal. Threshold levels, feedback inhibition and other factors are considered but not shown here. The pathological pathway of 20 subjects is illustrated. All sequential steps require a normal or pathological response (e.g. amplified, not diminished) so that the effector cell (step 6) is driven to cause a pathologic effect. Some states (e.g. A at step 3, or A3) are sensitive to one input and have one target, while others (e.g. B3) are sensitive to multiple inputs and affect multiple downstream states. Note that within a population there are some common pathways (1C-2A-3A-4B-5B-6 and 1D-2C-3C-4D-5C-6). Note also that a single factor (i.e. a single state) cannot be identified which *defines* the link between normal and pathology. Finally, some states (e.g. 3A) are only important connections if specific upstream and downstream risk factors are present (e.g. exposure to alcohol). A good clinical model will predict the likelihood of a subject progressing to pathology under a variety of conditions – and predict effective interventions. Figure © David C. Whitcomb. *Organizing known processes related to acute pancreatitis.*

environmental factor 1C. The study reporting a strong effect would not be reproduced. Does that mean that a report of a genetic association on one study is invalid? We would argue that it is not invalid.

Determining functional elements relevant to pancreatic diseases

Our overall pancreatic disease model suggests that a destructive, complex disorder can only progress from normal tissue to end-stage disease if there is either a combination of genetic defects, overwhelming environmental insults or pathologic gene-environmental interactions *at every step.* If this is true, then we must approach complex disorders differently than we do single-agent disorders, such as an infectious disease. A better study design might be to isolate factor 3C from Figure 6, selecting at all subjects that have reached step 2, and determining if there is an effect of factor 3C in proceeding to step 4. If there is, then the connections between the specific elements in these steps should be determined. The number of subjects needed for the first experiment could be relatively small ($<<1000$ subjects) since the variance is markedly reduced compared to a model in which end-stage disease is compared with non-disease controls. The number of subjects necessary to study the connection between steps is even less, because the probability that any one person would have an uncommon risk factor in two or three consecutive steps is very small.

Additional models for the analysis of complex disorders

We have outlined many of the reasons that a systematic approach is needed for understanding complex disorders. Once the key components are organized into a logical series, then formal modeling of the disease process can be applied, tested, additional experimental data added, the system calibrated and

retested. The optimal model or models are yet to be determined experimentally, but comparisons between simulations and experimental data must be tested. For example, logistic regression models can be applied our data to determine the attributable risk for each exposure. A highly sensitive procedure for identifying population substructure has also recently described by Pritchard et al. [85], in which several markers (or exposures) are evaluated for their ability to partition the sample into distinct groups using a Monte Carlo Markov-Chain (MCMC) method. New Bayesian-based models are also being developed for better computational modeling [86, 87]. The final question, however, is whether these new approaches will provide better health for our patients.

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