Review

Nutrigenomics in the whole-genome scanning era: Crohn's disease as example

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Abstract. Nutrigenomics has the potential to tailor diets to optimize health, based on knowledge of key genetic polymorphisms. Identification of candidate genes is often based on *a priori* knowledge of disease processes. However, genome-wide association methods are not only validating previously identified genes and polymorphisms, but also revealing new gene-disease associations not anticipated from prior knowledge. In Crohn's disease (CD), such studies not only confirm the importance of caspase-activated recruitment domain 15 and major histocompatability complex II molecules, but also reveal strong associations with the proinflammatory cytokine interleukin-23 receptor and autophagy-related 16-like gene. Genes identified to date in CD can be linked into two interrelated pathways: receptor-mediated cytokine induction or autophagocytosis. New genomic technologies need to be matched with innovative methodologies to characterize the likely impact of foods and to take the field to another dimension of value for human diet development and optimized health.

Keywords. Nutrigenomics, genome-wide association study, single-nucleotide polymorphism, Crohn's disease.

Introduction

Good nutritional advice for one individual may be inappropriate or even dangerous for another. While some of this variation relates to e.g. sex and exercise level, genotype is a major factor. The field of nutrigenomics utilizes new high-throughput technologies to provide detailed information about the composition and functions of the genome, mechanisms for regulation of gene expression, and the influence of nutrients on gene and protein expression [1]. In combination with the sister field of nutrigenetics, this field has the potential to lead to evidence-based dietary intervention strategies for maintaining health and fitness and preventing diet-related disease. Much of the early work in this field provided easily understandable examples. For example, single-nucleotide polymorphisms (SNPs) in the methylene tetrahydrofolate reductase (*MTHFR*) gene impact on folate metabolism and lead to significant differences among members of population groups in folate requirements, thereby affecting disease risk [2]. A considerable body of work by Ordovas and collaborators has considered the role of apolipoprotein E (ApoE) and ApoA1, interacting with intake of dietary lipids, in cardiovascular disease (CVD) [3, 4]. Ordovas points to the importance of SNPs in several genes that have differential effects on lipid metabolism. Mechanistic studies coupled with population data may

suggest that modifying the intake of omega-3 poly-unsaturated fatty acids (n-3 PUFAs) will be beneficial for some, but not all of the population. A Time article in 2006 [5] provides a popular-level summary, under the title, 'Does my diet fit my genes? The new science of nutrigenomics has some answers. It explains why fat and caffeine are worse for some than others.'

A recent review [6] recognizes the applications of the field to certain specific examples relevant to CVD or obesity and also identified some of the major reasons that this field has been slow to be translated into standard medical or dietetics practice. All health professions depend on a strong evidence base before taking largely research-based results into clinical practice. However, while we understand the role of some of the key genes in CVD, such as *MTHFR* or *APOE*, more solid information for other genes and/or other diseases, as well as genes associated with more nebulous concepts such as 'health,' 'wellness' or 'mood,' has been slow to accumulate. Two major stumbling blocks to date have been the time required to accumulate population data on the incidence of different SNPs and to prove definitively the significance of a given SNP in clinical disease, or other health parameters. These two factors provide a necessary first step in developing drug or diet treatment options for those individuals in whom the SNP is detected.

Significant advances in the technologies available to perform genomics studies, coupled with powerful international consortia combining patient databases [7, 8], have led to a rapid expansion of the information base available to nutrigenomic/nutrigenetics programs. The recognition that the risks of degenerative diseases, while having some genetic component, are largely attributable to diet, make these fields not just a luxury but also a necessity for future population health. In this review, we use Crohn's disease (CD) as an example to illustrate how this burgeoning information base can be combined with more traditional nutritional knowledge to begin to rationally design diets for specific purposes.

Identifying genes implicated in CD – early studies

Early observations of familial clustering of CD, and monozygotic twin concordance, led to the suggestion that this disease has a genetic basis. A range of methods including candidate gene studies, pedigree analysis, genotyping using microsatellite markers and linkage analysis by non-parametric allele-sharing methods provided supporting evidence for the genetic basis of the disease and identified many of the genes and chromosome regions of importance [9, 10]. On-line Mendelian Inheritance in Man (OMIM) catalogs

previous evidence on associations with susceptibility to CD that have been independently replicated [11]. The chromosomal locus on which the first key gene was characterized was the IBD1 region on16q12, where variants in caspase-activated recruitment domain 15 (*CARD15*) substantially enhance the risk of CD in several different populations [12–14]. We have also pointed to the importance of another pattern recognition receptor, toll-like receptor 4 (TLR4) in the disease [15, 16].

In addition to IBD1, OMIM also identifies IBD2 on 12p13.2-q24.1, IBD3 on 6p, IBD4 on 14q11-q12, IBD5 on 5q31, IBD6 on 19p13, IBD7 on 1p36, IBD8 on 16p (not linked to *NOD2/CARD15*), IBD9 on 3p26 and IBD10 on 2q37. The large IBD2 genomic region contains several possible candidate genes, including the transcription factor, *STAT6*, involved in the regulation of the T_{H1}/T_{H2} immune response [17]. This region, however, appears to be involved with the other form of inflammatory bowel disease (IBD), ulcerative colitis, rather than CD. The IBD3 locus on chromosome 6p, containing the major histocompatibility complex (MHC) region, has consistently shown linkage across a number of IBD association studies [18]. Among the genes likely to be affected are classes of human leukocyte antigen (HLA) genes that encode cell surface antigen-presenting proteins [16]. Duerr et al. [19] identified the IBD4 region in a genome scan using 751 microsatellite loci in 127 CD-affected relative pairs from 62 families. Suggestive linkage to the same locus was found in an independent study [20], although the key genes have not been adequately characterized. Pierik et al. [21] confirmed previous associations and showed a strong environmental interaction (especially cigarette smoking) with IBD4. The IBD5 region on 5q31 has been linked with CD in several studies, although there has not been agreement about the key genes in this chromosomal region [22, 23]. In particular, OMIM identifies mutations in two transporter genes, *SLC22A4* and the *SLC22A5* promoter, as possible causal variants [11]. Tello-Ruiz et al. [24] failed to determine the identity of the relevant gene(s) in IBD6, although they tested a considerable number of SNPs across 56 candidate genes in this region. CD linkage with a region of chromosome band 1p36, the IBD7 region, was reported in two different populations by Cho and coworkers [25, 26]. This region contains several possible genes of interest, including *EPHA2* (ephrin A2) which has been implicated in intestianal epithelial migration and barrier function [27]. Hampe et al. [28] identified IBD8 as a second susceptibility locus for IBD on chromosome 16p that was independent of *CARD15*, although again it was not possible to relate the signal to any particular genes. Three studies in

different population groups, using different methodologies [29–31] implicate chromosome 3p as important, with Duerr et al. [29] refining this region to 3p26 (IBD9), although again no convincing gene association has been suggested to date.

Linkage analysis has been a primary tool to identify chromosomal regions that cosegregate with the presence or absence of IBD in pedigrees with multiple family members with the disease. Brant and Shugart [9] and Daly and Rioux [32] review the strengths and weakness of this approach and of newer methods. One of the main problems with linkage analyses is that the method will only define fairly large chromosomal regions, and other approaches are necessary for establishing the genes of importance. Brand and Shugart [9] suggest that genome-wide association (GWA) studies with increased numbers of subjects and more comprehensive genomic coverage would provide the necessary new tool for understanding the genetic basis of many of the common human chronic diseases.

Identifying genes implicated in CD – GWA studies

Recent methodological advances make possible GWA studies, measuring large numbers of markers for genetic variation with sufficient power to detect statistically significant data. Particularly important has been the availability of dense genotyping chips that provide good coverage of much of the human genome at reasonable costs and that are technically simple to use. It has also been important to have phenotypically well-characterized clinical samples for CD. Several studies report data from sets of hundreds of thousands of SNPs, which combine to reveal information for thousands of well-characterized cases and controls [7, 8, 33–35]. These arrays, coupled with statistical methods for imputing information on genes not represented on the chip [36], provide a mechanism for unselected investigation of disease relationships, scanning across the entire genome. Decreasing costs and increasing sensitivity make these methods applicable to a larger number of samples from populations. Some of the reports recently available for CD provide information on up to 300,000 or 500,000 SNPs, and new arrays being released will increase those numbers. These higher-powered studies increase the probability of discovering genes associated with such complex diseases and have revealed some hitherto unexpected associations. Of particular importance is the large study by the Wellcome Trust Case Control Consortium (WTCCC) [8] that not only reports on approximately 500,000 SNPs in 3000 controls and 2000 CD cases from across

Britain, but also permits downloading and independent analysis of their dataset. The well-documented strength of association in these studies permits identification of 17 key regions of the genome with probabilities of $< 1 \times 10^{-5}$. The areas thus identified are shown in Table 1, along with possible genes and IBD regions from OMIM.

One major theme across many of the studies highlights the importance of specific components within innate and adaptive immune pathways, as has been previously predicted [16]. Duerr et al. [33] identified *IL23R* as an IBD gene. This gene on chromosome 1p31 encodes a subunit of the receptor for the proinflammatory cytokine interleukin-23 (IL23), and an uncommon coding variant was shown to confer strong protection against CD. The most important newly emerging theme from these recent studies on CD is the importance of defects in autophagy and the processing of phagocytosed bacteria (see below). Rioux et al. [7] and Hampe et al. [35] identified the IBD10 region and linked CD susceptibility to variation in the autophagy-related 16-like 1 (ATG16L1) gene. This gene encodes a protein in the autophagosome pathway. The WTCCC [8] confirms these two previous reports and identifies the strongest signal in their study as the *ATG16L1* gene. Their data also implicate several SNPs around the immunity-related guanosine triphosphatase gene (*IRGM*) as showing strong signals. This gene is again involved in elimination of intracellular bacteria, and encodes a GTP-binding protein which induces autophagy [37].

Linkage analysis versus association studies

Until recently, linkage analysis offered the only viable genome-wide method of identifying genes or chromosomal regions involved in a given disease. It is generally accepted that linkage analysis is best suited for identifying genes which underlie monogenic 'Mendelian' disease [38] and, although this approach has also been used to identify the majority of the 10 IBD regions catalogued in OMIM, has achieved only limited success for most common diseases [39]. Combined, the IBD risk variants identified by linkage analysis account for an excess risk to siblings of approximately two-fold, whereas the known total excess risk is approximately 30-fold [32]. In comparison, association studies have been shown to have considerably greater power than linkage analysis to detect the common variants with relatively modest effects on disease [40] thought to underlie most common diseases [41]. Historically, labor and expense has limited association studies to a candidate gene approach on genes with a biologically plausible role in

Table 1. Regions of the genome showing the strongest association signals for Crohn's Disease as identified by the Wellcome Trust Case Control Consortium (WTCCC) [8].

Chromosome	Region	IBD region (OMIM)	Gene in or closest to the region	WTCCC SNP	Trend p value	Genotypic p value
First-tier hits						
1p31	67.3–67.48		*IL23R*	rs11805303	6.5×10^{-13}	5.9×10^{-12}
2q37	233.92–234	IBD10	*ATG16L1*	rs10210302	7.10×10^{-14}	5.26×10^{-14}
3p21	49.3–49.87		*BSN MST1. USP4 GPX1 RHOA TCTA AMT NICN1 DAG1 APEH RNF123 AMIGO3 GMPPB IHPK1 LOC389118 C3orf54 UBE1L TRAIP]*	rs9858542	7.71×10^{-07}	3.58×10^{-08}
5p13	40.32–40.66		gene desert *PTGER4*	rs17234657	2.13×10^{-13}	1.99×10^{-12}
5q33	150.15–150.31		*IRGM MST150 ZNF300*	rs1000113	5.10×10^{-08}	3.15×10^{-07}
10q21	64.06–64.31		*ZNF365 C10orf22 EGR2*	rs10761659	2.68×10^{-07}	1.75×10^{-06}
10q24	101.26–101.32		*NKX2–3*	rs10883365	1.41×10^{-08}	5.82×10^{-08}
16q12	49.02–49.4	IBD1	*NOD2 NKD1 SLIC1 CYLD*	rs17221417	9.4×10^{-12}	4.0×10^{-11}
18p11	12.76–12.91		*PTPN2*	rs2542151	4.56×10^{-08}	2.03×10^{-07}
Second-tier hits						
1q24	169.53–169.67		*TNFSF18*	rs12037606	1.79×10^{-06}	1.09×10^{-05}
5q23 (5q31)	131.40–131.90	IBD5	*IL3 CSF2 P4HA2 PDLIM SLC22A4 FLJ44796 SLC22A5 LOC441108 IRF1*	rs6596075	5.40×10^{-07}	3.19×10^{-06}
6p22	20.83–20.85		*E2F3 CDKAL1*	rs6908425	5.13×10^{-06}	1.10×10^{-05}
6p21	32.79–32.91	IBD3	*HLA* (various)	rs9469220	8.65×10^{-07}	2.28×10^{-06}
6q23	138.06–138.17		*TNFAIP3*	rs7753394	4.42×10^{-06}	2.59×10^{-05}
7q36	147.62–147.70		*CNTNAP2 C7orf33*	rs7807268	6.89×10^{-06}	4.42×10^{-06}
10p15	38.52–38.57		*KLF6*	rs6601764	2.56×10^{-06}	8.95×10^{-06}
19q13	50.89–51.07		*RSHL1 SYMPK FOXA3*	rs8111071	6.14×10^{-06}	1.75×10^{-05}
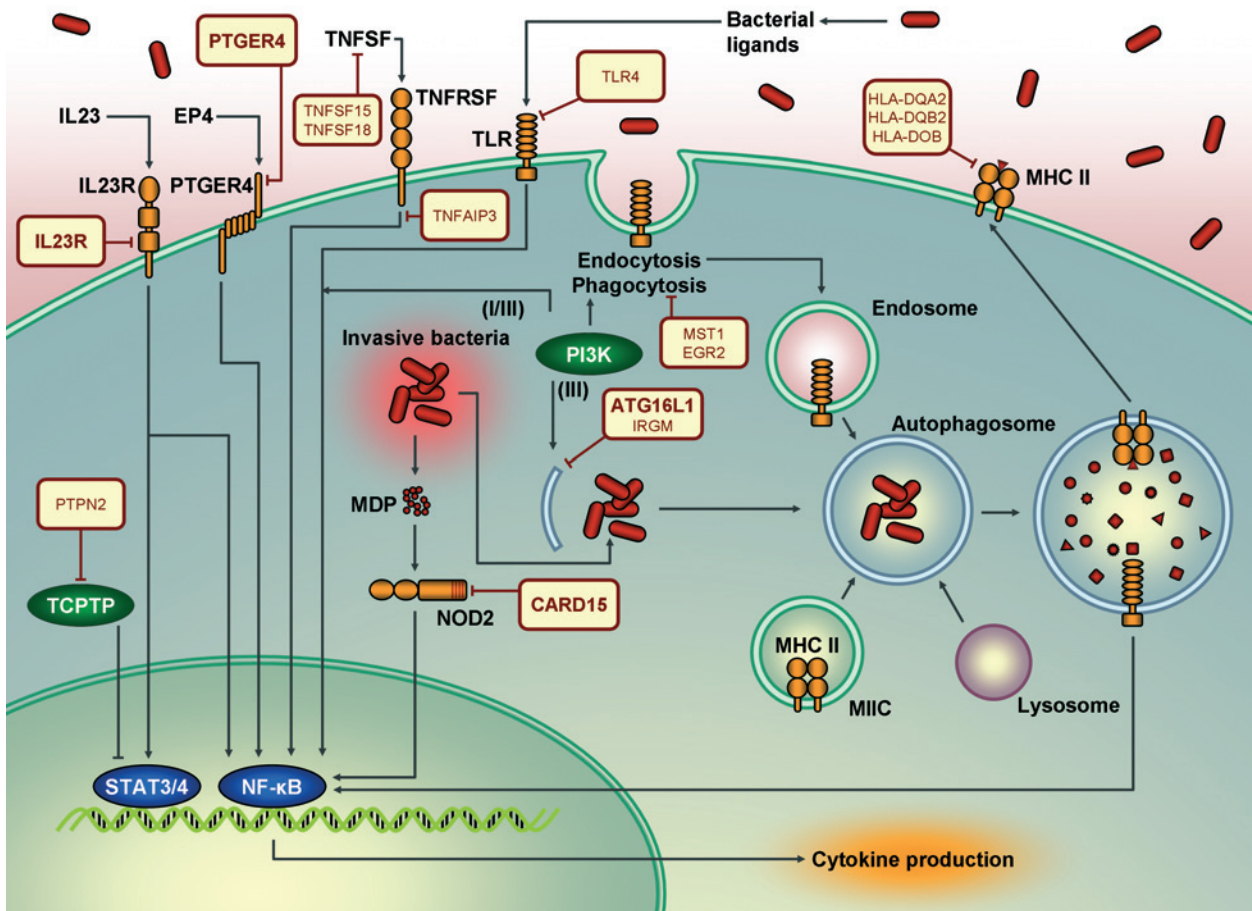
Figure 1. Possible interrelationships between the various genes implicated in CD by GWA studies. See text for a detailed explanation.

disease. However, the advent of high-density geno-typing chips allowing the interrogation of up to 500,000 SNPs has now made GWA studies possible. An example of the power of the GWA approach is the WTCCC study[8], which detected the OMIM IBD regions 1 and 10 at $p < 5 \times 10^{-7}$, IBD 3 and 5 at $p < 1 \times 10^{-5}$ and IBD 6 and 7 at $p < 1 \times 10^{-4}$, only failing to detect IBD 4, 8 and 9 at $p < 1 \times 10^{-4}$. In comparison OMIM lists only 4 of the top 17 hits ($p < 1 \times 10^{-5}$) of the WTCCC study. The failure of the WTCCC study to detect IBD 4, 8 and 9 may reflect differences in how linkage and association studies are powered, popula-tion differences, or in the cases of IBD 8 and 9, the fact that these regions have each only been reported by a single study [28, 29]. Thus, combined with the falling costs of the enabling technologies and increasing number of SNPs that can be interrogated, GWA studies are likely to become the mainstay for the discovery of genes and variants involved in common complex disease.

Likely functional roles of SNPs involved in CD as identified by GWA studies

In only a minority of the regions identified in whole-genome association studies has the biological mech-anism that leads to CD been clearly established. However, it is intriguing that candidate genes from all but one of the WTCC first-tier regions ($p < 5 \times 10^{-7}$), and many of the second tier regions ($p < 1 \times 10^{-5}$) (Table 1) can be linked to the two interrelated pro-cesses of receptor-mediated cytokine induction or endocytosis/phagocytosis/autophagocytosis (Fig. 1). Polymorphisms in *CARD15*, which encodes the pro-tein NOD2, were first related to CD by Hugot et al. [12] and Ogura et al. [14]. NOD2 is an intracellular receptor for the bacterial cell wall component mur-amyl dipeptide (MDP). Ligation of MDP to NOD2 leads to activation of the NF-κB pathway resulting in the production of proinflammatory cytokines [42]. Three coding SNPs (R702W, G908R and 1007fs) with increased prevalence in CD [12, 14, 43] have been shown to impair NOD2 function [44], and NOD2

knockout or transgenic mice have been shown to develop colitis upon bacterial challenge [45]. The most accepted mechanisms explaining this apparent paradox between loss of a proinflammatory signal and development of an inflammatory disease is that defective NOD2 impairs the initial innate immune response to bacteria, allowing the bacteria to proliferate, resulting in increased inflammation driven by the subsequent adaptive immune response. Alternative explanations have been reviewed by Strober et al. [46] and Hugot [47].

Polymorphisms in the region of the *IL23R* gene were first linked to CD in GWA studies. A coding variant (Arg381Gln) was found to confer strong protection against CD, while a number of non-coding variants were independently associated [33]. IL-23-driven production of the proinflammatory cytokine IL-17 by T cells has been shown to be a requirement for colitis in a mouse model [48], and blockade of the p40 subunit of the receptor, which is shared with the IL-12 receptor, has shown promising results in CD patients [49, 50]. Thus, loss of function mutations in IL23R would be expected to be protective against CD.

Libioulle et al. [34] identified variants in a 1.25-Mb gene desert in chromosome 5p13.1 that associated with CD. They showed that these variants correlated with increased expression levels of the nearest gene, *PTGER4*, which encodes the prostaglandin receptor EP4. Prostaglandin E4 is proinflammatory, triggering the activation of NF-κB, and knockout mouse studies have shown that *PTGER4* is involved in the development of colitis [51]. Prostaglandin receptor EP4 is also important in the activation of T$_{reg}$ cells [52–54] and disruption of this process could contribute to the loss of tolerance to commensal bacteria and to the inflammation that is the hallmark of CD.

Logistic regression analysis of variants in *ATG16L1* established by GWAs [7,35] showed that the coding variant A197T could fully explain the association signal to this locus and it was deemed to be a causal risk variant for CD [7]. *ATG16L1* is a key component in the formation of autophagosomes [55, 56]. Autophagy is a process whereby damaged organelles or proteins are encapsulated inside a double membrane and targeted for lysosomal degradation [57]. Recently, it has become clear that the autophagy pathway is also a crucial component of the innate immune system and is capable of capturing and degrading intracellular pathogens [57, 58]. RNAi knockdown of *ATG16L1* was shown to inhibit the autophagy of *Salmonella typhimurium* [7]. Thus, variants in *ATG16L1* may contribute to CD by impairing the ability of cells to destroy intracellular bacteria, allowing them to proliferate and subsequently generate an adaptive immune response.

Numerous other genes have been implicated in CD by whole-genome association studies and verified in replication studies (Table 1), although functional polymorphisms have not yet been demonstrated and tested experimentally for these candidate genes. However, adding weight to the likelihood that these genes are involved in CD is the fact that the majority of them are involved in the same processes as *CARD15*, *IL23R*, *PTGER4* and *ATG16L1* (Fig. 1). *IRGM* has been shown to induce autophagy in response to interferon-8 (IFN-γ) via a phosphoinositide 3-kinases (PI3K)-mediated pathway and thereby eliminate intracellular *Mycobacteruim tuberculosis* [37]. *MST1*, which encodes macrophage-stimulating protein, promotes phagocytosis via a PI3K-dependent mechanism [59]. Similarly, *EGR2*, which is one of the genes present in the 10q21 region identified by the WTCCC study and encodes early growth response 2, has been implicated in phagocytosis [60]. Loss of function in any of the interrelated pathways of autophagy, phagocytosis, or endocytosis (which can shuttle pathogen surface receptors such as TLR4 to autophagosomes, allowing them to respond to intracellular bacteria [61–63]) would impair the innate immune response, allow invading bacteria to proliferate and necessitate increased inflammation by the subsequent adaptive immune response.

PTPN2 encodes T cell protein tyrosine phosphatase (TC-PTP), an anti-inflammatory molecule that works by dephosphoylating and deactivating STAT3 [64] and STAT6 [65, 66]. Thus loss of TC-PTP function might be expected to exacerbate the proinflammatory effects of IL-23 mediated by STAT3.

A number of early association studies [18], as well as GWA studies [8], have linked CD with susceptibility alleles in the MCH region of chromosome 6. The HLA-D genes implicated encode MHC II molecules. Significantly, it has recently been realized that the process of autophagy allows the presentation of intracellular pathogen-derived antigens on the MHC II molecules of cells not considered to be professional antigen-presenting cells [67]. Thus, polymorphisms in HLA-D genes may disrupt this important bridge between the innate and adaptive immune systems.

TNFSF15 and *TNFS18*, members of the tumor necrosis factor (TNF) superfamily, encoding TNF-like 1 and glucocorticoid-induced tumour necrosis factor receptor family-related protein ligand, respectively, are proinflammatory cytokines whose effects are mediated through NF-κB activation, via pathways similar to TLRs and NOD2 [68, 69]. TNF-α induced protein 3 (TNFAIP) inhibits NF-κB activation [70] driven by TNF-α, and other TFNSF members [71]. These candidate genes have been identified by GWAs and, to date, the nature of any functional polymor-

phisms is unknown. However, their mechanistic similarities with TLRs and NOD2 provide a strong rationale for their involvement in CD. Similarly, KLF6 is a member of the Kruppel-like factor transcriptional regulators, which have been shown to modulate NF-κB activation, proinflammatory cytokine production and phagocytosis [72].

Nutrigenomics and CD

There have been a considerable number of studies on diet and CD, but none in which genotype has been simultaneously reported alongside dietary information. Removing CD patients from their normal diet and providing them with an elemental diet produces a significant reduction of symptoms in most, but not all, individuals. Although Woolner et al. [73] developed a low-fiber, low-fat exclusion diet (LOFFLEX) for use in CD, their practical experience is that this formula is really only effective after remission has been started with an elemental or oligopeptide formula. They currently advise enteral feeding until remission is apparent, followed by LOFFLEX for 2–3 weeks to confirm that the disease is under control, and then reintroduction of remaining foods singly to establish intolerances [J.O. Hunter, personal communication]. Although solid foods can be reintroduced, diets need to be individualized for such a dietary approach to enable continued remission from symptoms.

We have previously suggested that CD provides a good example in which both the incidence and severity of the disease could potentially be modulated through developing dietary recommendations according to genotype (nutrigenetics) [16]. Several of the genes now suggested to be involved in CD have been studied in model systems or in relation to other diseases, and some dietary interactions are known or implied. Some examples follow.

Pattern recognition receptors – *CARD15* and *TLR4*
Since both of these genes recognize components of bacterial cell walls, thereby affecting the immune response, it might be expected that modulation of the intestinal microflora could affect responsiveness to the normal form of the gene and SNP variants. Although probiotic therapy was considered to show promise for some individuals with CD, clinical trials suggest that those tested to date have limited efficacy, except for cases of pouchitis [74]. While probiotics may sometimes be beneficial for CD patients, it should be noted that Cukovic-Cavka et al. [75] attributed liver abscess in a *CARD15*-positive patient with CD to *Lactobacillus acidophilus*. In this example, they suggested the cause may have been immunologic incom-

petence due to corticosteroid treatment of CD. Prebiotics may be a more promising approach [76]. One of the reasons that elemental nutrition induces disease remission in CD may be through its capacity for modification of the gut microflora, possibly due to both its low-residue and also prebiotic properties [77].

Other dietary components may act more directly on the relevant gene products. Philpott and coworkers [78] described a cell culture-based assay which showed the ability of various food extracts, including those from kiwifruit and blueberries, to modulate the expression of NF-κB through *CARD15*. However, these extracts failed to restore the activity of the *CARD15* 1007fs variant to normal activity in this system. Borthakur et al. [79] showed that carrageenan, a high molecular weight sulfated polygalactan used widely within the food industry, induced IL-8 production through a distinct Bcl10 pathway in normal human colonic epithelial cells. They studied effects of a dose range of this material on the activity of several genes using immunohistochemistry, Western blotting, ELISA, and cDNA microarray techniques. Their results led them to suggest that exposure of human intestinal epithelial cells to carrageenan triggers a distinct inflammatory pathway via activation of Bcl10, involving NF-κB activation and upregulation of IL-8 secretion. Since Bcl10 contains a caspase recruitment domain similar to that found in CARD15, the authors suggested that carrageenan could be detrimental in individuals carrying variant SNPs in this gene.

As the receptor for lipopolysaccharide (LPS), TLR4 plays a critical role in innate immunity. Arbour et al. [80] showed that the common missense mutation (Asp299Gly) that we have associated with CD in human populations [15] affects the extracellular domain of the TLR4 receptor, and is associated with a diminished responsiveness to LPS in human cells. Their findings provided the first genetic evidence that common mutations in *TLR4* were associated with differences in LPS responsiveness in humans, and demonstrated that a common SNP altered the ability of the host to respond to an environmental stress. Lee and coworkers [81] provided evidence that a saturated fatty acid (lauric acid) and an n-3 PUFA (docosahexaenoic acid, DHA) reciprocally modulate the activation of TLR4 and its downstream signaling pathways. They suggested that TLR4-mediated target gene expression and cellular responses are also differentially modulated by saturated and polyunsaturated fatty acids. Sadeghi et al. [82] found that vitamin D3 [1α,25-dihydroxycholecalciferol, 1,25(OH)2D3] downregulated monocyte TLR expression and triggered hyporesponsiveness to pathogen-associated molecules.

All of the above studies used either the intact gene or a knock out model. It will be instructive to consider the effects of the polymorphisms found to be affected in CD on such results, and to consider the effects of the relevant nutrients in human epidemiology and/or clinical studies.

Interleukin-23

While specific *in vitro* or *in vivo* models for this disease have not been developed, some of the information on effects of dietary components in regulating gene expression in animal models may be relevant to developing nutrigenomic foods likely to have an effect in individuals who have high levels of expression of this gene. One of the most relevant reports and model systems may be that reported by Gremy et al. [83] who reported effects of caffeic acid phenethyl ester in modulating the T_{H1}/T_{H2} balance and cytokine pattern in the ileal mucosa of a rat after γ-irradiation. They showed that this chemical was able to downregulate IL-23, in a similar manner to that predicted by the human studies of the SNP modulating IL23R. The IL-10 knockout mouse model described by Roy and coworkers [84] provides a promising approach to identify other such candidate materials.

ATG16L1

The primary role of autophagy is adaptation to starvation at a cellular level, involving turnover of intracellular proteins and organelles and production of amino acids in situations of nutrient deficiency [85]. The pathway is activated as an adaptive response to a variety of environmental stress conditions, including nutrient deprivation, hormonal or therapeutic treatment, bacterial infection, and damaged organelles. Autophagy is thus heavily dependent upon the nutritional status of the cell and the tissue, independent of the degree of functionality of the relevant genes.

Kadowaki et al. [86] have reviewed the role of nutrients in the control of autophagy in mammalian cells. The availability of nutrients, especially amino acids, provides a key physiological regulator of this process. The effects of glucose and vitamins have also been studied extensively in the mammalian hepatocyte, which has been utilized extensively in mechanistic studies of autophagy. It is of some interest that these are also key nutrients identified in the dietary regulation of symptoms of CD. Scherz-Shouval et al. [87] provide evidence that reactive oxygen species (ROS) are essential for starvation-induced autophagy. They showed that starvation stimulates formation of ROS, specifically H_2O_2, and treatment with antioxidants abolished the formation of autophagosomes and the consequent degradation of proteins. Expression of this regulatory mutant prevented the forma-

tion of autophagosomes in cells, thus providing a molecular mechanism for redox regulation of the autophagic process.

Since downstream effects of the defective autophagy process appear to be important, either inhibition or enhancement of the process may provide an option. Benzoic acid, a weak organic acid food preservative, is an inhibitor of autophagy, at least in *Saccharomyces cerevisiae* [88]. Lavieu et al. [89] showed that knocking down the expression of the autophagy protein Atg7 by siRNA abolished starvation-induced autophagy and increased apoptotic cell death. In contrast, Gossner and coworkers [90] showed that genistein, a naturally occurring isoflavonoid abundant in soy products, increased autophagy in tumor cell lines. It is not known how cells carrying the CD variant SNP would respond to such a signal.

Although the variant *ATG16L1* genotype in CD alters the amino acid sequence and is assumed to impair normal protein function, it has not been investigated in as much detail as the Δatg7 cells studied by Onodera and Ohsumi [91]. They showed that under normal nutrient conditions, the total intracellular amino acid pool was reduced in the mutant cells, and the levels of several amino acids fell below critical values. In contrast, wild-type cells maintained amino acid levels compatible with life. Autophagy-defective cells fail to maintain physiologic amino acid levels, and their inability to synthesize new proteins may partly explain most phenotypes associated with autophagy mutants. This strongly validates approaches currently being used with CD patients using an elemental diet. It may also suggest other nutrients or approaches that could be useful in CD patients carrying this genotype.

MHCII and HLA

The CD regions identified by the WTCCC [8] span HLA-DP, HLA-DQ and HLA-DP, and partly overlap with celiac disease, which results in a permanent intolerance to gluten. Wheat gluten is a complex mixture of at least 100 related proteins [92] whose major components are the gliadins and glutenins, both of which can be subdivided into distinct protein families. Celiac disease is also triggered by related prolamins from barley and rye and is characterized by a strong association with *HLA-DQ2* and *HLA-DQ8* genes. Karinen et al. [93] showed that a gene dose effect of the *DQB1*0201* allele contributes to the severity of celiac disease (homozygous vs heterozygous). Similarities between the characteristics of certain CD patients and celiac patients have been noted since at least 1971 [94–96]. Excellent reviews of celiac disease pathophysiology are provided by Koning et al. [92] and Stepniak and Koning [97, 98].

Virtually all celiac disease patients share certain HLA-DQ molecules (HLA-DQ2 or HLA-DQ8), while dietary responses lead to specific antigenic peptides in the diet that are present in wheat, rye, and barley [96]. Tursi et al. [99] provided data to show a high prevalence of celiac disease among patients affected by CD, and indeed suggested that all CD patients should be started immediately on a gluten-free diet at diagnosis. James [100] showed the extent to which certain breakfast cereals, including wheat-based cereals, increased the probability of CD. These overlaps between celiac disease and CD could well relate to the HLA genotypes, although that question has not been formally investigated at this time. It should be noted, however, that celiac disease contrasts somewhat with typical CD in its morphological presentation and characteristics [101].

Dietary intolerances possibly associated with CD patients carrying SNPs in the HLA region. HLA molecules function to bind peptides and to present these to T cells. When such peptides derive from a pathogen, the T cells can mount a response that ultimately leads to pathogen eradication. In celiac disease, the immune system responds to HLA-DQ-bound gluten peptides, leading to inflammation and tissue damage in the small intestine and thereby to flattening of the intestinal villi. Indeed, celiac disease may be thus far the best-validated subject for nutrigenomics, where data accumulated since the early 1990 s have allowed deciphering of the interplay between the triggering environmental factor (gluten), the main genetic risk factor (*HLA-DQ2/8* haplotypes), and an autoantigen [enzyme tissue transglutaminase (tTG)]. More recent work suggests a contribution of innate immunity triggered by a distinct gluten peptide and driven by the proinflammatory cytokine IL-15. Together, these observations provide a unique explanation for the disease-inducing capacity of gluten [92]. A considerable amount of work relating colonic responses to specific structural characteristics of gluten proteins has enabled gluten-free foods, targeted to celiac patients and other sensitive individuals, to take a significant market share [97, 102–105].

Approaches used for developing and validating foods for celiac disease – lessons that could be applied to other diseases. The dietary treatment of celiac disease is based on lifelong withdrawal of foods containing gluten [106]. However, compliance with a gluten-free diet has proved poor in many patients, mainly because of their low palatability. Pizzuti et al. [106] used *in vitro* duodenum biopsies of celiac and control patients to identify cereal varieties that were not toxic to their patients. They compared immune responses to wheat (*Triticum aestivum*) with those to a traditional cereal (*T. monococcum*) on biopsies from 12 treated celiac patients and 17 control subjects, cultured for 24 h with gliadin from either cereal at 1 mg/ml. They were able to show that the more traditional cereal did not induce the same responses as did wheat gliadin. Such methods, however, could not be described as non-invasive.

The structural and chemical characteristics of foods that cause gluten intolerance can be studied in transgenic mouse models, providing much more flexible, rapid, and accurate answers than human studies. Senger et al. [107] identified immunodominant epitopes of α-gliadin after oral immunization in an *HLA-DQ8* transgenic mouse model. They expressed a recombinant α-gliadin (r-α-gliadin) in *Escherichia coli*. The transgenic mice were fed a gluten-free diet, then immunized with a chymotryptic digest of the r-α-gliadin along with cholera toxin as adjuvant. Spleen and mesenteric lymph node T cell responses were analyzed in an *in vitro* proliferative assay that tested the response to a panel of synthetic peptides encompassing the entire sequence of r-α-gliadin. This model of gluten hypersensitivity was able to show the structural characteristics of peptides that elicited an immunogenic response.

Once new bioactive compounds have been identified, incorporated into manufactured or functional foods and are being eaten by human populations, other methods can be used to validate their efficacy. Kemppainen et al. [108] considered local immunological response at the cellular level in adult celiac disease, after the individuals had been consuming oats for 5 years as part of their gluten-free diets. They were able to show that long-term use of oats in patients with celiac disease does not stimulate an immunological response locally in the mucosa of the small intestine. Ludvigsson et al. [109] considered the effect of *HLA-DQ2*, dietary history, and development of celiac disease on the induction of antibody response to wheat gliadin and cow's milk, β-lactoglobulin; IgG and IgA antigliadin, and anti-β-lactoglobulin antibodies were measured using enzyme immunoassay (EIA), and the effect of HLA risk genotypes, DQ2 and DQ8, on celiac disease were also considered. They showed an enhanced humoral response, not only to gliadin, but also to other food antigens.

Are coding SNPs the whole story?

The traditional understanding of the role of SNPs in disease susceptibility is that a coding SNP alters protein amino acid sequence, which in turn alters protein function, leading to altered gene expression

and cellular phenotype that may manifest as disease. Such a paradigm is demonstrated in CD by *CARD15*, where coding SNPs impair the ability of NOD2 to bind MDP [44]. Alternatively, the functional SNP may occur in a regulatory element of a gene, thereby altering transcriptional levels, as hypothesised for *PTGER4* [34].

While these traditional understandings of coding SNPs in cellular function are clearly still true, other regulatory mechanisms are becoming very apparent. Many of the regions identified in whole-genome association studies fall outside genes and regions normally associated with gene regulation. Indeed, *PTGER4* is one such example, with the associated SNPs falling in a 1.25-Mb gene desert upstream of *PTGER4*. Libioulle et al. [34] point out that a number of expressed sequence tags (ESTs) map to this region, suggesting the association could be with as-yet-unknown transcripts, such as small interfering RNAs (siRNAs). The likelihood that such non-gene-associated regions play important roles in disease is highlighted by the recent publication of the ENCODE pilot project [110], which showed that RNA transcript complexity is much greater than can be accounted for by known genes [111]. MicroRNAs (miRNAs) and siRNAs could account for some of these transcripts, but large intergenic transcripts of unknown function were also common [112]. Furthermore, some of the genes associated with CD, such as TLRs, have been shown to be epigenetically regulated [113]. Therefore, SNPs that alter methylation or histone acetylation patterns could alter gene expression. Such SNPs would not function in reporter gene assays or in transgenic animals, where epigenetic information is lost in the cloning process, but may still contribute to disease. Thus, the ability of whole-genome association studies to identify SNPs associated with disease may be outpacing the ability of biology to explain the causal effect, and the default assumption that intergenic SNPs are markers for functional SNPs in the regulatory or coding regions of genes may not always hold out.

In addition to known SNPs, copy number variants (CNVs) may be important in variations in gene expression that associate with disease [114]. Defensins protect the intestinal mucosa against bacterial invasion through the productions of endogenous antimicrobial peptides. Fellerman et al. [115] suggested that low copy number of the β-defensin-2, part of the β-defensin gene cluster on 8p23.1, predisposes to CD. It is also possible that where SNP analysis has failed to identify an important gene within other regions, CNVs could be responsible for the linkage data.

With the exception of altered patterns of methylation which may be affected by methyl donors in the diet, we do not at present have nutrigenomic approaches to link diet to these novel mechanisms of gene regulation.

Conclusions

Powerful new technologies have revolutionized our ability to associate diseases such as CD with quite specific areas of the genome, and specific genes in many cases. They overcome the need to utilize traditional linkage approaches to population studies, and the inevitable biases associated with candidate gene approaches. We are now in the position of recognizing many, if not most, of the key SNPs associated with human chronic diseases. In the example of CD, they provide a plausible set of two interconnecting pathways likely to be primary in the disease.

In contrast, the field of nutrigenomics, which will provide the ability to match dietary requirements to genotype, is still in its infancy. Sporadic reports suggest a number of dietary approaches that could link diet with genotype in a disease such as CD. These need to be systematically studied in a rational series of model systems, and validated in human epidemiologic or clinical studies. Only then will the field be in a position to reach its optimum potential.

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