ERC Advanced Grant 2016
Research proposal [Part B2] [1]
(not evaluated in Step 1)

Section a. State-of-the-art and objectives

Importance of the Project

The cause of Crohn's disease (CD) has been an enigma since its description over a century ago[1]. The disease has demonstrated a dramatic increase in incidence in developed countries in recent years so that now it affects roughly one in five hundred of the population[2]. It generally becomes apparent in the second and third decades of life and is a chronic debilitating condition that has a seriously detrimental effect upon the physical, social, sexual and professional lives of patients[3,4]. An estimated 2.5–3 million people in Europe are affected by IBD, with a direct healthcare cost of 4.6–5.6 billion Euros/year[5] of which about three quarters is contributed to by CD[6].
There has been a steep rise in the incidence of CD over the last few decades in economically advanced countries across Europe, North America and Australasia[7,8,9] . This is not purely an effect of increasing economic affluence because the incidence of CD is much lower in other economically advanced countries such as Japan and South Korea, although the incidence is now also rising in these countries[10,11].

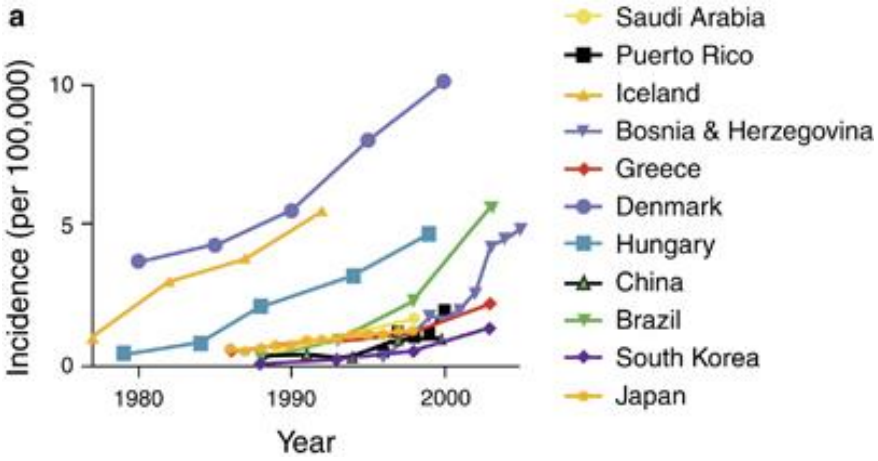


Figure 1. Increasing incidence of CD in several countries over time. Reproduced from [7]

CD is generally more common in urban females of higher socioeconomic status [9], with a female to male ratio of about 1.5-2:1.

Background

Infection has long been considered to cause CD. Several prospective studies have followed the course of patients after infections with enteric organisms and all have found an increased incidence of IBD as compared with uninfected control subjects[12,13,14,15,16]. In one of these[15] the risk of developing IBD after infection was similar whether or not a viable infecting agent was identified, suggesting that it was the damage to the bowel rather than a specific infection that was important. In the search for possible causal infectious agents, stool samples from CD patients have been extensively cultured and examined without a positive result (see for example[17]).This is not entirely surprising because the average time from the onset of symptoms to diagnosis of CD is over six months[18] by which time an infectious organism will have been eliminated if it was a triggering agent rather than the cause of a chronic infection. With the advent of next generation 16S rRNA gene sequencing the phylogeny and taxonomy of samples from complex microbiomes can be determined without the need for them to be viable or culturable. Dysbiosis of the faecal microbiome is well recognised in CD[19,20], with a decrease in the abundance and diversity of the *Firmicutes* phylum and an increased abundance of *Proteobacteria*, and alterations in the fungal composition[21]. Differences were also found between the microbiota of CD patients with ileal and with colonic disease[22]. This could reflect an epiphenomenon secondary to the disease process.

[1] Instructions for completing Part B2 can be found in the '*Information for Applicants to the Advanced Grant 2016 Call'*.

Major alterations in the microbiota are induced by diarrhoea[23], enteral nutrition[24], antibiotics[25], which most of these patients receive[26] and by iron therapy[27] which is often prescribed because these patients are generally anaemic. In general, the gut and mouth microbiomes display universal dynamics, unlike microbial communities associated with certain skin sites that are probably shaped by differences in host environment[28].

The considerable increase in the incidence of CD in developed countries in recent decades[29] has been attributed to immunological changes secondary to alterations in the environment (the Hygiene Hypothesis[30]). According to this theory, poorer standards of hygiene in lower socioeconomic societies lead to a greater abundance and variety of gastrointestinal pathogens. This would lead to a high incidence of gastrointestinal infections in infancy and childhood, resulting in death[31] or immunity[32]. CD is uncommon in underdeveloped societies in Asia[33], South America[34], China[35] and sub-Saharan Africa[36] and the increase in its incidence is closely associated with the improvement in income and living standards. Enteric infections are endemic in these developing societies in which diarrhoea is a major cause of death in children less than 5 years of age[37,38,39].

The population in lower socioeconomic status societies also host a large burden of gastrointestinal helminths[40] which have been theorised to protect from CD [41]. Helminthic infection was found not to be protective against CD in Denmark[42] and the outcome of several trials of iatrogenic infection with helminths as therapy for CD are awaited, but current evidence does not suggest that they will be efficacious[43].

If we postulate that the trigger for CD is enteric infection, the fact that the incidence of food-borne gastroenteritis which is fairly steady in most developed countries[44] must be reconciled with the rapidly increasing incidence of CD. Due to greater regulation and control of food production and distribution, the incidence of foodborne outbreaks of disease have remained steady or have declined[45,46,47]. Although enteric infections are generally considered to be foodborne, only about one half are in fact transmitted in this way[48], most of the rest being transferred by person to person contact.

It is important to consider the age distribution at which patients present with CD. It rises to a peak at between 20 and 30 years of age after which it demonstrates a steady decline, a pattern that is remarkably consistent, and very different from that of UC, across the geographical spectrum[11,49,50,51].

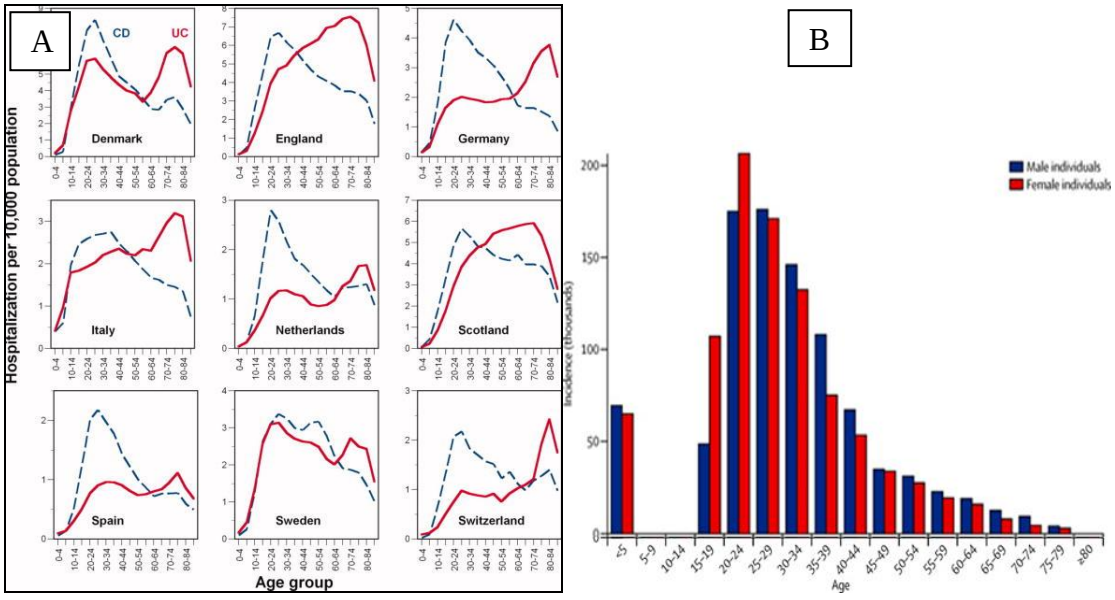


Figure 2
(A) **The age distributions of Crohn's disease and ulcerative colitis in several European countries.** (Reproduced from[49]) (B) **Global age-sex distribution of new HIV infections** [52]

The peak incidence, generally at a later age than puberty, coincides with a stage in life accompanied by major lifestyle changes. These include the movement of individuals out of the family home, in which the ambient microbiome is likely to be relatively stable, into environments in which the risks of exposure to infection are much greater. The main two ways in which young

adults are exposed to infectious enteric organisms is through the ingestion of contaminated food or fluids, or by person to person contact, the risk of both being increased by travel to places where exposure to novel organisms is more likely.

Sexual transmission

Sexual transmission is worthy of consideration as a means of transmission of faecal organisms between individuals. As might be expected if this were to be the case, the peak age for the acquisition of sexually transmitted diseases is very similar to that of CD (Figure2).
Epidemiological studies from developed countries have reported an increasing prevalence of invasive infections by *Entamoeba histolytica*[53], *Shigella*[54],*Cryptosporidia*[55] and *Campylobacter* [56], among men who have sex with men (MSM), which is not surprising because of the increased risk of exposure to coliform organisms by oral, anal and oro-anal sexual practices[57].
Given that oral and oro-anal sexual practices have been demonstrated to be responsible for the transmission of enteric infections in MSM, they must also pose a risk in the heterosexual population[58,59]. Amongst heterosexuals, anal sex is practiced by 30 - 40% of the population in England and North America, and fellatio and cunnilingus are almost universal[60,61]. In England the participation in anal sex has almost doubled over the last three decades, a similar increase to that of the incidence of CD. In terms of absolute numbers, approximately seven times more women than men engage in unprotected receptive anal intercourse[62]. In addition, the ratio of homosexual to bisexual men is about 3:1, and the latter can act as “bridgers”, transmitting infections from men who have sex with men into the heterosexual community[63].

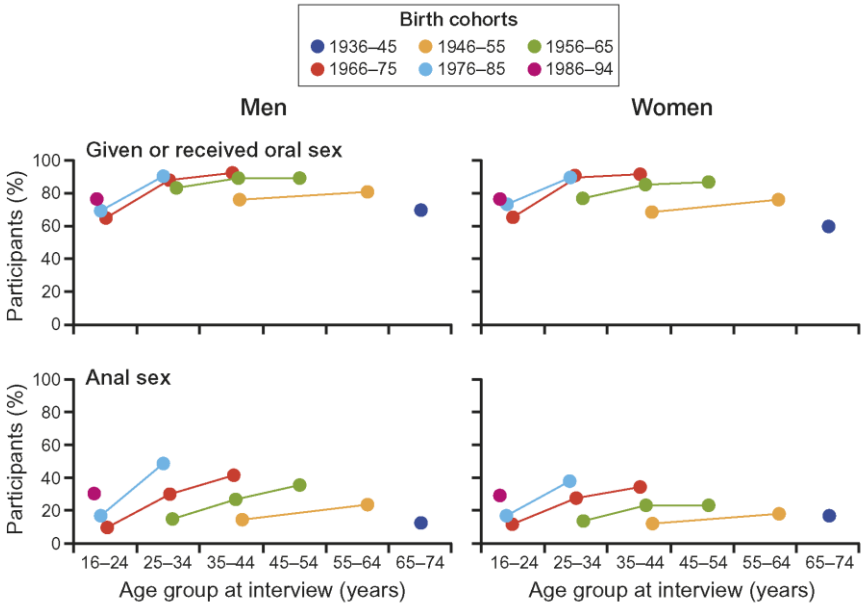


Figure 3 Heterosexual sexual practices in Britain. Redrawn from from the three National Surveys of Sexual Attitudes and Lifestyles[60]. Each line connects values for the same birth cohort at different ages

Those countries with a high standard of living and low rate of CD such as Japan[10], Taiwan, China[64] Korea[11], Saudi Arabia[65] and Malasia,[66] appear to have low rates of heterosexual anal sex http://www.data360.org/pdf/20070416064139.Global%20Sex%20Survey.pdf [67] and in these countries the sex ratio of the disease which is about 1.5-2:1 female:male[9] in Western countries, is reversed, implying that men are particularly vulnerable to infection in these places. This does not appear to be due to a reporting bias because the sex ratio of UC in these countries matches that of Europe and North America.

Immunoparesis of the acute inflammatory response is the underlying Crohn’s phenotype

Over the past forty years we have established the underlying, and unifying, predisposition to the development of CD as a systemic incompetence of the acute inflammatory response, resulting in a delay in the accumulation of neutrophils at sites of acute inflammation, and the failure to adequately eradicate bacteria gaining access to the tissues[68–73]. The delay in the recruitment of neutrophils by the innate immune response to sites of trauma to the body has been demonstrated in patients with CD in several different but complimentary ways. In 1976 I demonstrated that the accumulation of neutrophils in superficial abrasions on the arm, was grossly deficient when

compared with healthy subjects or patients with another chronic inflammatory condition, rheumatoid arthritis[68].
The next in these series of experiments was conducted on the ileal and rectal mucosa, and again on the skin[69]. A small mucosal biopsy was taken from the ileum or rectum, and this was then followed six hours later by a re-biopsy of the previous biopsy site, to determine the extent of the inflammatory response induced by the initial biopsy trauma. Once again there was a major delay in the recruitment of neutrophils in CD, and this was observed in both regions of the bowel. In addition to healthy subjects, control individuals with UC in remission were studied and their neutrophil recruitment was normal. Trauma to the skin reproduced the impaired neutrophil recruitment, as well as reduced secretion of IL-8 and IL-1β, from superficial abrasions. In normal subjects the direct injection of heat killed *E.coli* into the subcutaneous tissues of the forearm was followed by profound rise in local blood flow that was substantially impaired in CD, but not in UC[69]. Blood flow is important in recruiting innate immune cells to sites of inflammation and this already paltry vascular response in CD would be further compromised by smoking tobacco[74].
In the third of these experiments we directly measured the accumulation of neutrophils at the site at which *E.coli* had been injected subcutaneously, and the rate of clearance of these organisms[70]. In this study peripheral blood neutrophils were labelled with the gamma-ray emitting radioisotope Indium-111[75] and reinjected intravenously at the same time that unlabelled *E.coli* were injected subcutaneously into the forearms. The rate of accumulation of the radioactive neutrophils over the injected bacteria was determined and a much smaller proportion of neutrophils were recruited to the injected bacteria in the CD subjects than in the HC or UC individuals[70]. In the same study *E.coli* were radiolabelled with Phosphorus-32, heat killed, and injected subcutaneously. The clearance of these organisms was measured with an overlying Geiger counter. The clearance of bacteria was dramatically slower in CD.

The abnormality of innate immunity in most cases of CD lies in the macrophages

Defective secretion of pro-inflammatory cytokines in CD may be the explanation for the observed impairment in neutrophil recruitment[70,76,77,78,79]. In CD, the neutrophils themselves are normal[80] and exhibit normal migration *in vitro*[68,81,82] and will migrate out of skin windows if chemoattractant substances are placed over them[69]. In some very rare conditions with a CD-like phenotype, for example chronic granulomatous disease (CGD), the neutrophils are defective[83]. In the absence of a primary abnormality of neutrophil function, macrophages from most cases of CD showed defective secretion of pro-inflammatory cytokines, but normal release of chemokines, in response to stimulation with *E.coli*[70]. The genes for these pro-inflammatory cytokines were transcribed and translated, but the proteins were directed to lysosomal degradation rather than secretion, suggestive of disordered vesicle trafficking.

What is the molecular cause of the impairment of acute inflammation?

There is a strong genetic component to the aetiology of CD. The sibling recurrence risk (ratio of risk of disease manifestation, given that one's sibling is affected, compared with the disease prevalence in the general population) is approximately 13-36[84] and approximately 12%[85] of CD patients have at least one affected first degree relative. Furthermore, the study of over 300 twin pairs has demonstrated a higher concordance of disease phenotype in monozygotic (30%) compared with dizygotic twins (4%)[86]. While the twin studies support the role of genetic susceptibility, they also indicate the requirement for additional environmental or other factors, such as an enteric infection, for the development of overt disease.

How do we find these genetic factors?

*Linkage and Genome Wide Association studies (GWAS*Linkage

Linkage analysis of affected sibling pairs with CD permitted the identification of a susceptibility locus on chromosome 16 (termed *IBD1*) in which mutations in the gene *NOD2* were subsequently identified[87,88]. *NOD2* mutations remain the most strongly associated common genetic variants associated with CD. Other causal variants have not been identified by linkage; numerous factors can limit the effectiveness of linkage analysis such as the presence of unaffected individuals that harbour the mutation (incomplete penetrance), individuals who develop the disease as a result of mutations in another gene, or due to environmental factors (phenocopies), the requirement for the combined effect of two or more mutations (epistasis), or the requirement of the involvement of some environmental factor such as an infectious trigger (which will effectively reduce penetrance

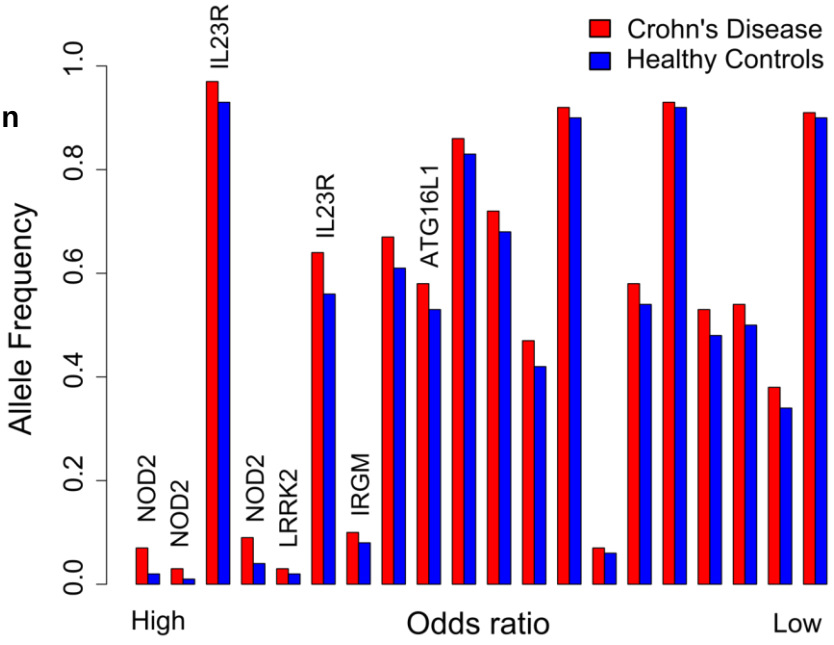
by not facilitating the manifestation of the underlying genetic predisposition in unexposed individuals).

GWAS

Genes reside on chromosomes which undergo recombination at meioses. Population level haplotypes arise due to the non-random positioning of crossing-over events. Haplotypes are characterised by a particular set of SNP genotypes. Depending on the ancestral origin and frequency with which a mutation has arisen in the population, it may occur on a particular haplotype and thus the SNP genotypes defining that haplotype will be enriched in patients harbouring the disease-causing mutation. Therefore, when comparing a large population of diseased individuals with healthy controls, SNPs tagging the underlying mutation should be enriched in the affected, compared with unaffected, individuals. In GWAS, a set of SNPs are genotyped in an attempt to cover the whole-genome and the above comparison made[89]. One of the major problems with analysing many hundreds of thousands (or millions) of markers across the genome is that the large number of comparisons undertaken risks producing false positives. This necessitates the utilisation of a stringent p-value threshold for significance of $p<5\times10^{-8}$ [90]. As a result very large sample sizes are required[91,92].

The GWAS approach has the advantage of being comprehensive (compared with candidate gene studies) and objective (at least up until the stage of data interpretation). There are however a number of limitations, for example incomplete genomic coverage. In addition, a major drawback is that the SNPs employed as markers must be relatively common in the general population, in order to give the study adequate statistical power, so this approach is typically unable to identify low frequency mutations, however penetrant or important.

When a SNP is found to be statistically significantly associated with a disease by GWAS, it can be because the polymorphism is itself pathogenic or, more commonly, that it is tagging a closely located genetic variant whose genotype correlates with that of the tagging polymorphism (the two variants are in linkage disequilibrium). Increasingly large GWAS have been performed on CD and the results meta-analysed[93,94]. No single, or small number, of penetrant mutations have been found that independently cause the disease. The latest study of over 20,500 CD cases and 41,600 controls of European ancestry identified 145 loci associated with CD at $p<5\times10^{-8}$. The mean OR of the SNPs representing these 145 loci was 1.16 and the mean control allele frequency was 0.48. Four SNPs had an OR exceeding 1.5 of which three were within *NOD2* and the fourth was in *IL23R*. The mean difference in allele frequency between cases and controls was only 0.02.[95]



in

Figure 4 The allele frequency Crohn's disease patients and healthy controls for the top 20 CD associated GWAS SNPs sorted by odds ratio. The data were taken from the European cohort in [96]. Loci harbouring genes of interest have been indicated.

The very significant p-values obtained for the associated loci, led to the general perception that the molecular causes of CD have been identified. Individually the CD GWAS loci have very modest effect sizes (i.e. a small difference in frequency in the control and CD populations), consistent with a polygenic model in which it is thought that it is the combination of these minor influences that is

causally important. However, in the latest meta-analysis, all significantly associated loci combined account for only 10.9% of the disease "heritability"[93].

An important consideration is that about half of the healthy population also carry these variants, although (by definition) each at a slightly lower frequency than in the CD patients. The healthy controls carrying these variants greatly outnumber the patients with CD in the population (Figure 4). With a prevalence of CD of about 2-3 patients in 1000[2], and taking the NOD2 frameshift mutation as an example because it has the greatest effect size at 3.32, for every 100,000 individuals in the population there will be 99,680 unaffected individuals of whom ~2390 will carry this mutation. In this population there will be ~320 CD cases of which ~48 will have the mutation[94]. This means that the penetrance of this mutation, with by far the greatest association with the disease, is only 2%. These effect sizes pale into insignificance when compared with the effect size of HLA-B27 in ankylosing spondylitis of approximately 94[97,98], and HLA in type 1 diabetes and coeliac disease with effect sizes of approximately 25 and 50, respectively[99].

GWAS conducted on Europeans and East Asian populations have yielded noticeably different findings. The significant heterogeneity in common variant CD genetic architecture between different populations provides a further indication that the genes identified by GWAS are unlikely to play a primary causal role in the development of the disease, the manifestations of which are similar in patients regardless of their ethnicity.

It is important to highlight that many of the associated genes common to these conditions have been implicated in pathways leading to activation or regulation of the immune response[100].

Macrophage expression profiling

In the knowledge that the release of pro-inflammatory cytokines by macrophages from CD subjects is depressed as a result of impaired vesicle trafficking[70], we attempted to identify genes contributing to this deficiency by looking for outlier levels of gene expression in these cells. The most commonly under-expressed gene was Optineurin (OPTN) which was under expressed in 10% CD patients studied[72].

Optineurin

Macrophages from patients with low expression of OPTN secreted abnormally low levels of pro-inflammatory cytokines, as do macrophages from OPTN knock out mice[71]. mRNA expression levels of these cytokines were normal, consistent with deranged secretion rather than synthesis. These mice were more susceptible to infection with *Citrobacter, E. coli* and *Salmonella*[101], and showed reduced levels of TNFα in their serum, diminished neutrophil recruitment to sites of acute inflammation and greater mortality, than wild-type mice. OPTN-knockdown zebrafish infected with *Salmonella* also had a higher mortality[71].

Optineurin (OPTN) is a ubiquitously expressed protein with different functional domains and a plethora of binding partners through which it regulates multiple cellular processes, including vesicular trafficking, inflammatory signaling, antiviral immune responses and mitosis[102]. In addition it has a role in autophagy, and cytosolic *Salmonella* were shown to be eliminated through an OPTN dependent xenophagy response[103].

DNA sequencing

The development, availability and reducing costs of high throughput DNA sequencing, has provided the means of directly identifying causal mutations in human disease[104,105]. Several studies employing such technology have been undertaken in CD[106,107] and many more are likely to appear over the ensuing years. High-throughput DNA sequencing has also been absolutely crucial for the diagnosis of the rare primary immunodeficiencies[108] (that produce bowel inflammation, rather than CD).

The major problem in identifying causal genes by sequencing is the considerable individual variation in DNA sequence. Asymptomatic individuals carry, on average, approximately 100 genuine loss of function variants with ~20 genes completely inactivated[109]. This makes it very difficult to identify the disease causing mutation/s in any one individual. Of note, an ongoing study in which whole-genome sequencing has been undertaken in 2,697 CD cases and 3,652 healthy controls failed to identify a single variant at genome-wide significance that had not already been identified by GWAS[110].

Alternative approaches have been taken to overcome the difficulty of the interpretation of individual variation. Several studies have focussed on the analysis of Ashkenazi Jews (AJ) because they

have a roughly fourfold increased incidence of CD and demonstrate genetic homogeneity having arisen from approximately 350 individuals about 30 generations ago[111].
Chuang et al. sequenced the exomes of 50 AJ CD patients and prioritised low frequency coding variants which were then genotyped in approximately 3,000 AJ CD cases and 3,000 controls. In a paper in which we participated they identified a frameshift mutation in *CSF2RB* as a strong causal candidate which was associated with CD at $p<3.5\times10^{-6}$ and an OR of 1.5[112]. This variant is rare in the non-AJ population.

We utilised a family based approach.

We characterised two very large AJ families with >800 and >200 members with 54 and 26 affected cases respectively, sequenced the exomes of all cases and imputed the genotypes of the unaffected family members[73].

In the larger family **(Family A)** we identified the same frameshift mutation in *CSF2RB* that was independently reported in sporadic AJs by Chuang et al[112]. Another strong candidate gene was *NLRP2*, a NOD-like receptor and a component of the inflammasome.

In the smaller of these families **(Family B)** we identified a damaging missense mutation in *DUOX2* (P303R) that impaired the function of the protein and showed a possible epistatic interaction with NOD2.

We have also collected, curated, and exome sequenced 350 sporadic AJ CD cases (and 600 cases from our small AJ CD families) which we have contributed to a consortium studying AJ CD centred at the Broad Institute in which exome sequences from approximately 2,500 cases and a similar number of controls have been collected and analysed. In addition to the known mutations in *NOD2*, some of the variants that we found in our data were nominally significant in the Broad Consortium data when comparing CD and control AJs, as shown in the table below. These include mutations in *CSF2RB* and *NLRP2* described above, the same mutation in *HEATR3* that has previously been described in association with AJ CD[113] and one in *LRRK2* that is located in a risk region for Crohn's disease[114]. We are co-authors on two papers that have been submitted for consideration for publication, one from the Broad consortium (submitted to *Nature Communications*) and another from Inga Peter (submitted to *Science Translational Medicine*), linking mutations in *LRRK2* to CD.

Gene	Consequence	Broad OR	Broad p	Broad HC	Broad CD	UCL CD
NLRP2	p.Glu522Gly	46.1	4.66E-06	0	0.004	0.038
HEATR3	p.Arg642Ser	2.0	1.70E-04	0.011	0.018	0.013
LRRK2	p.Asn2081Asp	1.4	3.01E-04	0.057	0.079	0.061
TNS1	p.Arg260Pro	2.0	7.62E-04	0.009	0.014	0.015
CSF2RB	p.Ser709LeufsTer22	1.5	9.47E-03	0.020	0.023	0.025

Table 1. Variants in 350 sporadic AJ CD patients that achieved statistical significance in collaborative Broad study.

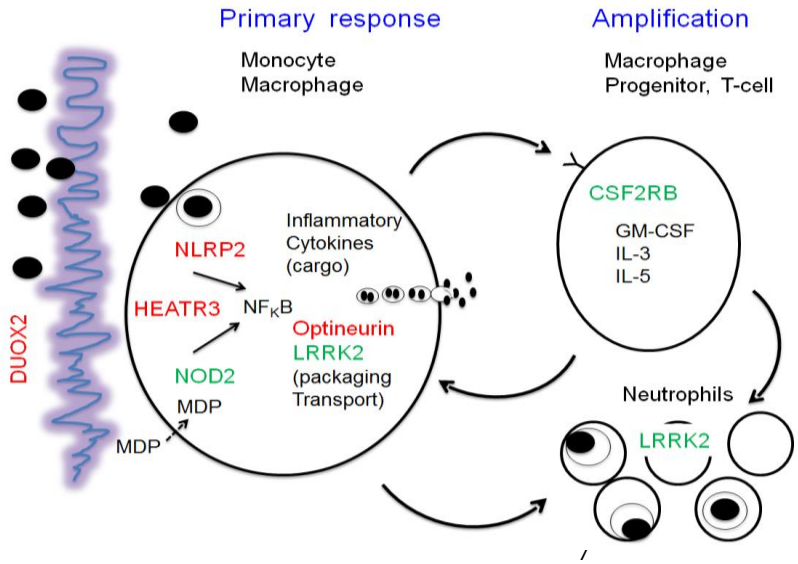


Figure 5 Indicating the molecules we have prioritised for analysis because of their involvement in inflammation and immunity, and their known (green), or likely (red), association with CD.

We will concentrate on the further analysis of the molecules highlighted in Figure 5 (Tensin 1 will not be taken further at this stage). I will briefly summarise the key points relating to these molecules:

NOD2

NOD (Nucleotide-binding Oligomerisation Domain) 2 is a member of an extended family of inflammatory and immune proteins in plants (the resistance (R) genes[115]), Drosophila (Toll-like receptors[116]) and animals (NOD families). These proteins combine a central nucleotide-binding domain (NOD) with a C-terminal leucine-rich repeat (LRR) motif and an N-terminal caspase recruitment domain (CARD) or equivalent.
In general these proteins recognise a signal from an invading organism in their LRR domain that induces a polymerisation that triggers a signalling cascade which terminates in the production and release of pro-inflammatory molecules. NOD2 is activated by muramyl dipeptide (MDP) a component of the cell wall of both Gram negative and Gram positive bacteria. It seems to be taken into the cells within endocytic vacuoles; presumably the organisms are then digested within this compartment and the solubilised MDP is moved into the cytoplasm by peptide transporters like *SLC15A3* [117]. Very recently it has been demonstrated that NOD1 and NOD2, do not only respond to bacterial stimuli, but are also important mediators of ER-stress-induced inflammation[118].
This theoretical model of NOD2 function as a pattern recognition receptor capable of inducing pro-inflammatory cytokine secretion has been validated by *in vivo* studies in humans in which the application of MDP to skin windows induced the production and release of pro-inflammatory cytokines in healthy and CD patients without NOD2 mutations, but not in those carrying the CD-associated mutations[69]. The impaired secretion of these inflammatory mediators into skin windows in the absence of MDP, in CD patients without mutations in NOD2 in this study, demonstrates that other pro-inflammatory signals and pathways must be abnormal in these subjects, suggesting that there are at least two parallel routes initiating the inflammatory response.

CSF2RB

Having been identified independently by two groups *CSF2RB* must be considered as a causal gene for CD in AJs. CSF2RB is the common or shared β subunit of the receptors for granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-3, and IL-5[119]. The distinct α chains of these receptors provide cytokine specificity whilst the β chain is responsible for high-affinity binding, and is the major downstream signalling component of the receptor complexes.
GM-CSF is produced by myeloid cells, dendritic cells (DCs), T cells, B cells, and several non-immunological cells including epithelial cells[120] following exposure to inflammatory stimuli to promote the production and function of myeloid haemopoietic cells including haemopoietic progenitor cells and differentiated cells such as basophils, neutrophils, eosinophils, macrophages and certain dendritic cells[121] to deal with the cause of the inflammation.
IL-3 is predominantly produced by activated T cells, natural killer (NK) cells and mast cells. It acts on the early stages of haematopoiesis in synergy with other cytokines to induce progenitors of various lineages but it is a very important stimulus for the generation of mast cells and the regulation of mast cell function as well as basophil production and activation.
IL-5 stimulates mainly the production and function of eosinophils. The major source of IL-5 is T-cells with relatively lower amounts produced by mast cells and eosinophils[122].

NLRP2

NACHT, LRR and PYD domains-containing protein 2. A member of the family of cytoplasmic proteins--the NALPs—that form a large, signal-induced multiprotein complex, the inflammasome, resulting in the activation of pro-inflammatory caspases in response to activation of Toll-like receptors (TLRs) during the cell's response to microbial infection[123]. Caspase-1 activation processes precursor interleukin-1β (IL-1β) and IL-18 into their mature bioactive fragments[124]. Most of the knowledge of inflammosomes comes from studies on NLRP3. Virtually nothing is known about the biochemistry and cell biology of NLRP2, but by analogy it is likely to be activated by TLRs and to have pro-inflammatory activity.

LRRK2

A considerable amount of work has been done on this protein because mutations in LRRK2 cause familial and sporadic Parkinson´s disease (PD). It is a large, 280kDa, protein with GTPase and kinase domains, the latter being constitutively active in PD. It is found in immune cells, in lamina propria macrophages, B-lymphocytes, dendritic cells, and neutrophils, and levels are markedly increased in the bowel in CD[114] and in microglia in the nervous system[125]. It interacts with small GTPases including Rab32 and Rab38 with which it co-locates to transport vesicles and recycling endosomes[126] and it is important for the elimination or intracellular *Salmonellae*[127] and *Legionella*[128]. The association of variants in *LRRK2* with CD gives added support to the observation of abnormal vesicle trafficking in macrophages from patients with this condition[70]. Rab32 and Rab38 play an important role in the biogenesis and traffic of melanosomes and lysosomes and this system is disordered in Hermansky-Pudlak syndrome[129], accounting for the characteristic partial albinism. If LRRK2 and its associated proteins are important for immunological resistance to the development of CD then it might be expected that CD would be more common in conditions in which the LRRK2 system is disordered, which is in fact the case - a clear association exists between CD and PD,[130,131] and CD and Hermansky-Pudlak[132].

HEATR3

Very little is known about this molecule which has previously been linked to AJ CD[113] (there are only 3 references to it in PubMed). It is highly expressed in mouse myeloid cells and had been linked to NF_KB activation CD[113], a pathway of established importance in CD.

OPTN

Optineurin (OPTN) is a ubiquitously expressed 67 kDa protein. Its expression can be induced by TNFα and interferons, probably as a result of NFκB activation, and it is localised in the cytosol and Golgi apparatus[102,133]. In essence it is a linker, or adaptor, molecule and has several binding partners including Rab8, Huntingtin, the gene that is mutated in Huntington disease, and Myosin VI, a multifunctional motor protein. Rab8 is a small GTPase involved in vesicular trafficking between the trans-Golgi network (TGN) and the plasma membrane. The function of Huntingtin itself is unknown but it is associated with several factors involved in vesicle trafficking. Myosin VI is attached by OPTN to the Golgi apparatus and then it participates in the transport of vesicles and their protein cargos from the from the Trans-Golgi network to be released at the cell surface. OPTN also contains a ubiquitin binding domain with the ability to bind polyubiquitinated cargoes and transport them to autophagosomes via its microtubule-associated protein 1 light chain 3-interacting domain[134].

DUOX2

We identified a damaging missense mutation in DUOX2 (P303R) that impaired the function of the protein and showed a possible epistatic interaction with NOD2. DUOX2 is a member of the large NADPH oxidase (NOX) family of enzymes[135,136] I initially discovered[137]. Its expression in the bowel epithelium is induced by the microbiota[138]. It generates H_2O_2 at the mucosal surface and this acts as substrate for lactoperoxidase catalysed oxidation of thiocyanate to microbicidal hypothiocyanite[139]. It might also attract neutrophils to inflammatory sites[140]. Knockdown of the DUOX2 homologue in invertebrates and mice resulted in an impaired tolerance to enteric bacteria[141]. Of relevance, given the possible NOD2 epistatic interaction observed, a physical and functional interaction between these proteins has been demonstrated in epithelial and HEK293 cells[142].

All these molecules relate in some way to immunity and inflammation. DUOX2 protects the mucosa against infection and might recruit neutrophils. NOD2 is a receptor for microbial products which also indirectly activate NLRP2, and both these molecules induce inflammation. OPTN and LRRK2 are similar in that they interact with RAB proteins, and are involved in vesicle and granule transport, cytokine secretion and degranulation, which we have shown to be deranged in CD[70]. CSF2RB is important in myelopoesis, and in amplifying the inflammatory response.

Principle objectives:

1. *Investigate the possible sexual transmission of enteric infections leading in some cases to CD.*

The epidemiology points to the possibility that the triggering enteric infection could be sexually transmitted in a proportion of cases. Once this possibility has been raised it is important to determine the public health risks of these sexual practises in relation to CD, and more broadly to the health of the general population. If such risks were identified they could be reduced by public awareness.

2. *Further the analysis of the large AJ CD families to identify asymptomatic carriers and phenocopies, the macrophage phenotype and their microbiome.*

These two families are by far and away the largest identified with CD, and some of the biggest employed in genetic studies. Their detailed analysis will provide further important insights into the genetic influences on the causes of CD, and this will be greatly strengthened by a more rigorous definition of their phenotypes.

3. *Validate and determine the modes of action of candidate variants in CSF2RB, DUOX2, NLRP2, LRRK2 and HEATR3 by generating and analysing knock-in and knock-out mutant mice.*

It is important to determine whether the variants that we have identified as associated with CD in our patients predispose to bowel inflammation in animal models. We will cross relevant knock-in and knock-out mice with NOD2 KO mice to determine whether these signalling pathways work in parallel or in series.

4. *Identify the components of the signalling pathways in macrophages by investigating molecules interacting with NOD2, OPTN, NLRP2, HEATR3 and LRRK2.*

We will attempt to define the signalling pathways and molecular interactions in which these molecules are involved upon macrophage activation with different agonists. We will then search for GWAS hits and variants in these associated molecules in CD patients. This should provide a more complete picture of these signalling pathways.

5. *Investigate the function of DUOX2 in mucosal immunity and its interaction with NOD2.*

The DUOX2 system is clearly important for defence of the gastrointestinal system from the mouth to the anus, and we know very little about it. We will quantitate H_2O_2 generation by, and mucosal microbial growth in, mucosal biopsies individuals carrying the DUOX2 (P303R) mutation. We will examine the biochemistry and cell biology of wild-type DUOX2, and of the (P303R) knock-in mutation in mice.

Section b. Methodology

Aim 1: To determine whether there is evidence of sexual transmission of CD

i. Are sexually transmitted infections (STIs) more common in patients with CD?

We will use STIs as indicators of sexual promiscuity, which could link with the increased risk of transmitting enteric infections sexually. We have access to the National Health Service Clinical Practice Research Datalink containing the general practice and hospital records of 2-5 million people. This work will be done in collaboration with Professor Harry Hemmingway (http://www.farrinstitute.org/). We will examine these records for evidence of a relationship between CD and STIs, either clinical infections, relevant tests, or test results.

In addition, we currently have blood samples from 700 patients with CD and will recruit a further 300 subjects to make up the numbers to 1000. We will analyse a similar number of control samples. We will test the sera for previous infections with *Chlamydia*[143], HSV type 1and 2, and papilloma viruses[144].

ii. Is Crohn's disease more common in individuals attending STI clinics?

This is planned to be a separate but complimentary study funded by alternative sources. We will prepare a very simple questionnaire to accompany the normal medical records in which patients attending STI clinics will be asked whether they have had Crohn's disease, ulcerative colitis or irritable bowel syndrome (as one group of controls). These clinics at the three participating hospitals, UCH (Dr Nigel Field), The Royal Free (Dr Dan Ivens) and St Mary's (Dr Alan Winston) see 150,000 patients a year. We should obtain sufficient data in one year to produce statistically significant results when compared with the General Practice database.

Professor Caroline Sabin will collaborate in the epidemiological and statistical analysis.

iii. Questionnaire of sexual practises amongst CD patients and controls

We have access to large numbers of patients with IBD: 3,000 at UCH and the Royal Free Hospitals; 5,000 at St Mark's Hospital (where we are collaborating with Professor Ailsa Hart), and 1,000 at the Homerton University Hospital. We will ask as many as possible of these patients, and an age and sex matched control cohort of patients attending the hospitals for other reasons, to complete questionnaires on their sexual habits. The design and analysis of the questionnaires will be done in collaboration with Professor Anne Johnson, who has great expertise in this subject having led the British National Survey of Sexual Attitudes & Lifestyles[60]. (https://iris.ucl.ac.uk/iris/browse/profile?upi=AMJOH29).

Control groups will be age and sex matched subjects attending hospital for other reasons, and we will also compare CD patients with those with UC. The effect of the disease on sexual activity will have to be taken into account and questions should also involve patterns preceding the onset of the disease.

If this pilot study gives any indication of the possibility that sexual habits might be associated with the triggering of CD we will extend it through Crohn's and Colitis organisations like Crohn's and Colitis UK (https://www.crohnsandcolitis.org.uk/), possibly by way of online surveys.

Aim 2: Further characterisation of the AJ CD families

2a. The two very large families

In order to strengthen the genotype/phenotype correlation, we will first attempt to identify endophenotypes and phenocopies in the two families, the inclusion or exclusion of which would strengthen the genetic analysis. Faecal calprotectin[145] and bowel permeability[146] (identified by standard oral lactulose/mannitol excretion sugar test) that are elevated in bowel inflammation, will be measured in as many affected and unaffected family members as possible. Where the diagnosis is uncertain, more direct diagnostic procedures will be performed where feasible.

We will measure cytokine secretion and mRNA transcription by monocyte derived macrophages from patients and unaffected family members in response to heat killed *E. coli* and a panel of TLR ligands[70].

Where possible we will perform skin window and bacterial injection tests on the subjects[69], to determine the inflammatory response *in vivo*.

In an additional attempt to identify phenocopies in the families we will closely question spouses marrying into the family to determine whether they themselves have a family history of IBD, and determine their calprotectin levels. A significant number of these spouses have already advised us as to the presence of IBD in their families and we will attempt to obtain DNA from affected and unaffected individuals in these associated families.

In the light of the DUOX2 mutation, in Family B we will measure H_2O_2 generation by mucosa obtained from rectal biopsies from patients[147]. Western blotting and immunohistochemistry will be used to determine tissue levels and cellular and subcellular distribution of the DUOX2 protein (good antibodies are available). In all cases patients will be compared with healthy controls and family members without the mutation. DUOX2 is thought to sterilise the overlying mucus layer, which is usually devoid of bacteria. We will determine whether the DUOX2 mutant individuals, CD and carriers, have an increase in the organisms in the mucus layer[148,149].

A number of studies have described an association between disease associated polymorphisms and dysbiosis of the gastrointestinal tract in Crohn's patient[150,151]. We will characterise the microbiota in affected and unaffected individuals in both families. In Family A this could explain the difference in the prevalence in the subfamilies, and in Family B it will show whether or not the mutation in DUOX2 has an influence on the microbiota. Bacterial DNA samples will be isolated and the microbiota determined using 16S ribosomal metagenomic sequencing[152] (in collaboration with Professor Mathias Chamaillard, University of Lille, CNRS, INSERM, Institut Pasteur de Lille).

2b. Multiple smaller families and sporadic cases.

We have identified 24 smaller AJ CD families with at least 4 affected members and 200 families with 2 or 3 affected. In addition we have contributed 350 sporadic cases to the Broad AJ IBD consortium. Most of these cases have been sequenced and we are currently analysing the results. I have set up two gastroenterology Fellowships in collaboration with Eran Israeli at Hadassah

Hospital in Jerusalem, funded by the Charles Wolfson Charitable Trust. These Fellows will each spend one year in Israel identifying CD families. One fellow will concentrate on Haredi AJs because they have large families like the two we have described, the other will concentrate on Druze and Muslim CD patients because they have a high proportion of consanguinity, which facilitates the identification of causal variants. The Druze communities in northern Israel have 40% first cousin marriages. The second year will be spent in my laboratory in London, sequencing the DNA and analysing the results.
Candidate variants will be identified and published but will not be further characterised at this stage. It is probable that some of these variants will be in molecules that are identified as associated with macrophage signalling pathways in the experiments described in Aim 4, below, providing mutually supportive evidence of their involvement in CD.
These studies will determine whether or not the candidate mutations that we have identified as contributing to CD predispose to bowel inflammation. In addition they will use molecules known, or strongly suspected to be associated with CD, to define the signalling and subsequent processes that lead to pro-inflammatory cytokine secretion. This should enhance our understanding of important physiological processes, whilst at the same time identifying molecules and pathways worthy of close attention for their potential involvement in the Crohn's process.

Aim 3: Assessment of function of CD candidate variants

3a: Generation of knock-in/knock-out mice

CD patients and families are genetically heterogenous and obtaining blood and tissue samples is often difficult and unreliable. We will thus reproduce the natural candidate variants in mice through a collaboration with Professor Werner Muller, University of Manchester.
We will make the mutations to *NLRP2* and *HEATR3* shown in Table 1. The *DUOX2* P303R knock-in is already in progress. With regard to *LRRK2*, as described below, we are in a position to analyse the impact of the mutations on function in transfected cells. In addition, our collaborator Professor Dario Alessi (MRC PPU unit, Dundee) already has mutant mice with activating and inactivating *LRRK2* mutations.
The *CSF2RB* gene is duplicated in the mouse, which will make genotyping difficult and raises the possibility of redundancy or compensatory effects. We will therefore knock-out this duplicate gene before knocking in the human variant.
LoxP sites will be inserted into these constructs so that a complete KO can also be generated if required. Because of the possibility that NOD2 is involved in a parallel, accessory pathway to that activated by Toll receptors, promising mouse models will also be tested on a $NOD2^{-/-}$ background to look for complementarity of the signalling pathways.

3b: Studies on mutant mice

i. Susceptibility to gastrointestinal inflammation

It is important to identify, and concentrate on, the functional variants that predispose to bowel inflammation. We run several different mouse models to investigate gastrointestinal inflammation. Colonic inflammation will be induced through the exposure of mice to 2% DSS for seven days, oral gavage of *Citrobacter rodentium* or *Salmonella typhimurium*[71,153]. We will also use a mouse model of acute ileitis induced through the oral administration of the parasitic protozoan, *Toxoplasma gondii* (ME49 strain), as previously described[154]. Bowel inflammation and systemic alterations will be determined through the use of multi-parameter FACS analysis of the leukocyte population within the gastrointestinal tract, changes in tissue and serum cytokines, histological scoring of the tissue, bacterial translocation to the spleen and lymph nodes, changes in body weight, bowel length and mortality.
DUOX2 is only expressed in the gastrointestinal tract and the experiments on this molecule will focus on the animal model rather than isolated cells. We have identified a damaging missense mutation in (P303R) that impaired the function of the protein and showed a possible epistatic interaction with NOD2[73]. The mutant mice will allow us to directly measure rates and extent of H_2O_2 generation[147] at different regions of the bowel, both by explants, by inverted sacs, and by perfused, vascularised, intestinal loops in situ[155]. We will be able to determine how these change with bowel inflammation and what effects DUOX2 has in the attraction of neutrophils[140].
We will characterise the microbiota in the mucus layer[148,149] and faeces of the mutant and wild type littermate controls (mutant and WT will be co-housed from birth in order to reduce the effect of environment on any dysbiosis.).

ii. Immunological studies

The composition of the immune system will be analysed by flow cytometry and histology. We will determine the differential cellular composition of the bone marrow, blood and primary and secondary lymphoid organs. Immunological studies into the adaptive immune system in these mice will be conducted by Professor Werner Muller. Functional immunological investigations will be conducted if and when indicated.

iii. Cellular studies: Signalling and trafficking

Prior knowledge indicates that abnormalities in signalling pathways (for example NOD2), or the machinery making or secreting inflammatory cytokines, leads to the CD phenotype, and we will concentrate on exploring these processes.

Signalling:

The common CD phenotype is the impaired secretion of pro-inflammatory cytokines by macrophages of these patients. Signalling pathways must be important for cytokine secretion, and this fact is underlined by the fact that NOD2 is a signalling molecule, and its impaired function predispose to CD. In addition, several of our candidate molecules, LRRK2, NLRP2 and HEATR3 are to a greater or lesser extent known to be involved in signalling.

NOD2 is clearly involved in the signalling response to bacterial products. The crystal structure of NOD2 has been solved and it showed a direct interaction with MDP[156]. We also know that a number of downstream signalling molecules, including RIPK2[157], are modulated by activated NOD2. We do not know the accessory and adaptor molecules that are directly associated with NOD2 or all the downstream effector molecules that link to the activation of NF_KB.

Similarly, impaired Il-8 and IL-1β secretion from skin-windows in CD can be corrected by the application of MDP, but only in patients with wild-type NOD2. Therefore there are clearly additional and complimentary signalling pathways to macrophage activation. It is possible that it is necessary to have the failure of a critical signalling molecule, or the combined failure of both the NOD2 and an alternative pathway, to produce the impairment of macrophage function that causes the CD phenotype. This would explain why even homozygote frameshift mutations in NOD2 have little effect in the majority of individuals.

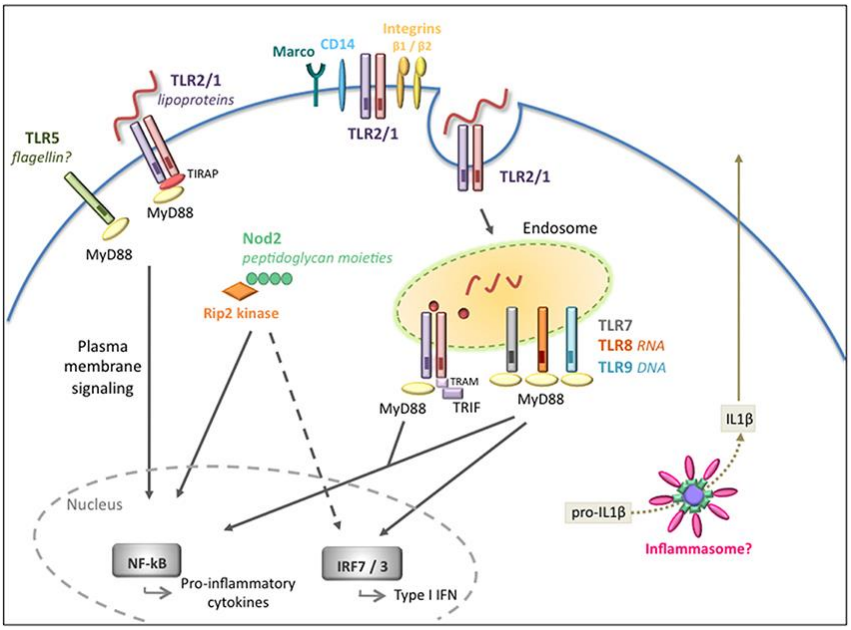


Figure 6. Bacteria induced signalling to an inflammatory response in macrophages. From [158].

This figure illustrates that there are multiple parallel signalling pathways involved, one of which is the NOD2 mediated response to MDP. Lesions in several of these pathways would be required to produce a major impairment of the inflammatory response.

Trafficking and autophagy:

We have shown that macrophages from CD patients transcribe and translate TNF, but divert it to lysosomal degradation rather than secretion after exposure to heat-killed *E.coli*. We also know that impaired expression of OPTN leads to a similar secretory defect. OPTN has been identified as a linker, or adaptor, molecule with a number of well characterised binding partners which combine to transport intracellular vesicles.

Several of the GWAS loci contained molecules identified as associated with autophagy, in which tissues are remodelled and damaged or effete organelles and proteins removed. To undertake this process, cells must first identify the target for engulfment by labelling it with ubiquitin[159,160], the ubiquitinated material is then encircled by a double membranous structure, the autophagocytic

vacuole that then fuses with lysosomes containing enzymes that digest the inner membrane and its contents[161]. What is probably of particular relevance to CD is the specialised type of autophagy, known as xenophagy, that is designed to deal with bacteria that escape into the host cytosol (such as *Shigella* or *Listeria*) or those that reside in a modified intracellular vacuole (such as *Salmonella* and *Mycobacteria*), which is important for the entrapment and lysosome-mediated degradation of bacterial pathogens[162,163]. LRRK2[114] and OPTN[102,133] have both been shown to be important for autophagy.

3c: Development of cell based systems

Initial studies will be performed on bone marrow derived macrophages purified from the mutant mice. Subsequently we will work on cultured cells to reduce the numbers of mice experimented upon and to facilitate molecular manipulation. We will use two types of cell lines:

1. The THP-1 human leukaemia monocytic cell line, which has been extensively used to study monocyte/macrophage function[164].

2. Immortalized monocyte derived macrophage cell lines from the mutant mice, and wild type controls, that we will generate using the methodology developed by Wang et al[165]. These cell lines will then be maintained in culture and differentiated into mature macrophages using M-CSF. This methodology has been shown to be a suitable alternative to bone marrow derived macrophages.

We will then use a lentivirus-based CRISPR/Cas9 system[166] to create stable mutations in these cell lines. We have already made NOD2 mutations in THP-1 cells, a global knock-out, a frameshift (3020insC) associated with CD, two activating mutations (R334W, R334Q) that produce Blau syndrome[167] and we are currently knocking-out RIPK2.
It is highly plausible that CD associated molecules do not work in isolation and the combination of numerous proteins cooperate and interact resulting in increased susceptibility to disease. In order to test this we will use our transgenic macrophage cell lines and introduce additional mutations in candidate proteins through the use of gene editing (CRISPR/Cas9). Double/Triple mutant cell lines will then be screened for alterations in cytokine secretion, autophagy induction and inflammasome activation.

Assays of cell function

Cytokine expression and secretion

The cells will be stimulated with MDP, LPS and other TLR ligands, and live and heat-killed *E.coli*, and the induction of pro-inflammatory genes quantified by qPCR. The secretory levels will be determined using Meso Scale Discovery multiplex cytokine assays.
Intracellular location and trafficking of cytokines will be visualized and characterised through the use of GFP-cytokine constructs and confocal microscopy. Intracellular trafficking compartments will be identified using antibodies against well-recognized markers (eg EEA1, GM130).

Signalling pathways

The initial studies will involve the determination of phosphorylation of macrophage proteins after stimulation with the ligands described above. Initially we will determine time courses and overall patterns with Western blots using anti-phosphotyrosine, threonine and serine antibodies. Once the parameters have been defined our primary experimental technique will be quantitative mass spectrophotometry (in collaboration with Professor Alexander Schmidt, Biozentrum Basel). We will use SILAC[168] to quantitatively differentiate the compared cell lines and will attempt to measure the proteome-wide dynamics of phosphorylation and ubiquitylation.
We will investigate the mutant **NLRP2** on the activation and regulation of the inflammasome which will be measured through the cleavage of Caspase 1 and the release of IL-1[169].The formation of the inflammasome complex will be visualize and monitored using a GFP tagged adapter protein apoptosis associated speck-like protein containing a caspase activation domain adapter termed ASC. ASC helps the formation of a multi-protein complex which contain all the inflammasome components and precedes IL-1processing[170].The ASC-specks can be observed as they reach a size of around 1 µm and can be used as an indicator of inflammasome activation[171].
LRRK2. Naturally occurring *LRRK2* mutations in the CD patients will be tested for functional effects in collaboration with Professor Dario Alessi by co-transfection of HEK293 cells with *LRRK2* and *RAB10*. LRRK2 activity causes the phosphorylation of RAB10 and of Ser935[172] of LRRK2 that

can be identified by Western blotting, the former in a Phos-tag (Wako) gel retardation assay.
This will allow us to determine an increase or decrease in the activity of any of the LRRK2 mutants we detect in our patient populations.
HEATR3 activity will be determined by the effects of the mutation on I_KB degradation, NF_KB phosphorylation at Ser536[173] and cytokine secretion.

3d: Protein/protein interactions

The majority of the molecules which we have identified with the exception of OPTN are poorly characterised in respect to their role in the immune response, protein complex composition, binding partners and regulatory domains. In order to gain a greater understanding of how these molecules work and what influence the Crohn's disease variants have on these processes we will utilize a number of complementary methods.
We will use a proximity-dependent biotin identification (BioID) technique. This is a recently developed method that allows the identification of proteins in the close vicinity of a protein of interest in living cells. It relies on fusion of the protein of interest with a mutant form of the biotin ligase enzyme BirA (BirA*) that is capable of promiscuously biotinylating proteins located in close proximity to the fusion protein[174]. Biotinylation of proteins in close proximity to NOD2 will be identified by transfecting the NOD2 deficient cells with biotinolase A (BirA*) fused to wild-type NOD2, constitutively active, and inactive NOD2. Other molecules of interest, such as OPTN or NRLP2 will be treated in a similar way. The stably infected cell line will be stimulated with MDP, LPS or live or heat-killed whole bacteria, and biotinylated proteins identified by mass spectrometry.
As an additional means of identifying binding partners we will also perform immunoprecipitations of our target proteins in cells in the resting and activated states. They will be tagged with the HaLo-tag[175] which is also fluorescent allowing subcellular localization confocal microscopy.
When we have delineated the functions of candidate molecules and the mutations in them, in the Knock-in and KO mouse models, and in cell lines, we will then go back to the patients and confirm that the mutations that they carry produce the same biological effects (as we have done for OPTN[72]). The molecules in the signalling pathways and vesicle transport systems that we identify will be compared with the GWAS hits and variants in the Broad and other Crohn's and colitis databases. This will allow us to develop an understanding of the normal physiological processes and mutations that compromise their function.

Aim 4: Investigation of potential future treatments

The identification of proteins involved in the macrophage activation pathways will, in all likelihood, identify molecules, mutations of which predispose to CD. As a greater number of these predisposing, or causal, variants are identified, it will become feasible to develop diagnostic microarrays that will be helpful in identifying individuals at risk of CD that may be useful in prenuptial, preconception planning and in disease diagnosis.
Bone marrow transplantation can cure CD disease[176] but suitable donors are rare and the procedure carries a high mortality. The demonstration that in the majority of cases the macrophage is defective in its response to bacterial stimuli indicates that those cases should be amenable to gene therapy because macrophages in the bowel are regenerated from blood monocytes[177] whose progenitor cells are present in bone marrow. Hence it should be feasible to aspirate marrow, purify the progenitor cells, transfect them with the correct gene, or edit out the mutation with CRISPR technology, and re-inject the cells into the conditioned patient. This has recently been shown to work with pulmonary macrophages in mice with alveolar proteinosis[178]. We will be in a position to test this in our mice.
The cause of CD has posed a major conceptual hurdle for over a century. The identification of the underlying phenotype as a failure of acute inflammation was a considerable advance. Now we are at the stage at which specific molecules and their biochemical and cell biological mechanisms can be delineated. The work described in his application will utilise multiple complimentary methodologies and approaches, facilitated by collaborations with experts across Europe, including epidemiology, genetics, biochemistry and cell biology studies on humans and animals, and on primary cells and cell lines, to define causal molecular mechanisms as the prelude to the development of specific diagnostic and therapeutic modalities.

References

1. Dalziel TK. Chronic intestinal enteritis. *BMJ*. 1913;ii:1068–1070.
2. Molodecky NA, Soon IS, Rabi DM, et al. Increasing incidence and prevalence of the inflammatory bowel diseases with time, based on systematic review. *Gastroenterology*. 2012;142(1):46–54.e42; quiz e30. doi:10.1053/j.gastro.2011.10.001.
3. Lönnfors S, Vermeire S, Greco M, Hommes D, Bell C, Avedano L. IBD and health-related quality of life - Discovering the true impact. *J Crohns Colitis*. 2014;8(10):1281–6. doi:10.1016/j.crohns.2014.03.005.
4. Abraham BP, Sellin JH. Disability in inflammatory bowel disease. *Gastroenterol Clin North Am*. 2012;41(2):429–41. doi:10.1016/j.gtc.2012.02.001.
5. Burisch J, Jess T, Martinato M, Lakatos PL. The burden of inflammatory bowel disease in Europe. *J Crohn's Colitis*. 2013;7(4):322–337. doi:10.1016/j.crohns.2013.01.010.
6. van der Valk ME, Mangen M-JJ, Leenders M, et al. Healthcare costs of inflammatory bowel disease have shifted from hospitalisation and surgery towards anti-TNFα therapy: results from the COIN study. *Gut*. 2014;63(1):72–9. doi:10.1136/gutjnl-2012-303376.
7. Talley NJ, Abreu MT, Achkar J-P, et al. An evidence-based systematic review on medical therapies for inflammatory bowel disease. *Am J Gastroenterol*. 2011;106 Suppl :S2–25; quiz S26. doi:10.1038/ajg.2011.58.
8. Economou, M. Zambeli EMS. Incidence and prevalence of Crohn's disease and its etiological influencesNo Title. *Ann Gastroenterol*. 2009;22:158–167.
9. Timmer A. Environmental influences on inflammatory bowel disease manifestations. Lessons from epidemiology. *Dig Dis*. 2003;21(2):91–104. doi:73242.
10. Asakura K, Nishiwaki Y, Inoue N, Hibi T, Watanabe M, Takebayashi T. Prevalence of ulcerative colitis and Crohn's disease in Japan. *J Gastroenterol*. 2009;44(7):659–65. doi:10.1007/s00535-009-0057-3.
11. Lee K-M, Lee JM. Crohn's disease in Korea: past, present, and future. *Korean J Intern Med*. 2014;29(5):558–70. doi:10.3904/kjim.2014.29.5.558.
12. Helms M, Simonsen J, Mølbak K. Foodborne bacterial infection and hospitalization: a registry-based study. *Clin Infect Dis*. 2006;42(4):498–506. doi:10.1086/499813.
13. García Rodríguez LA, Ruigómez A, Panés J. Acute gastroenteritis is followed by an increased risk of inflammatory bowel disease. *Gastroenterology*. 2006;130(6):1588–94. doi:10.1053/j.gastro.2006.02.004.
14. Ternhag A, Törner A, Svensson A, Ekdahl K, Giesecke J. Short- and long-term effects of bacterial gastrointestinal infections. *Emerg Infect Dis*. 2008;14(1):143–8. doi:10.3201/eid1401.070524.
15. Gradel KO, Nielsen HL, Schønheyder HC, Ejlertsen T, Kristensen B, Nielsen H. Increased short- and long-term risk of inflammatory bowel disease after salmonella or campylobacter gastroenteritis. *Gastroenterology*. 2009;137(2):495–501. doi:10.1053/j.gastro.2009.04.001.
16. Jess T, Simonsen J, Nielsen NM, et al. Enteric Salmonella or Campylobacter infections and the risk of inflammatory bowel disease. *Gut*. 2011;60(3):318–24. doi:10.1136/gut.2010.223396.
17. Van Kruiningen HJ, Colombel JF, Cartun RW, et al. An in-depth study of Crohn's disease in two French families. *Gastroenterology*. 1993;104(2):351–60. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8425677. Accessed February 26, 2016.
18. Maconi G, Orlandini L, Asthana AK, et al. The impact of symptoms, irritable bowel syndrome pattern and diagnostic investigations on the diagnostic delay of Crohn's disease: A prospective study. *Dig Liver Dis*. 2015;47(8):646–51. doi:10.1016/j.dld.2015.04.009.
19. Oberc A, Coombes BK. Convergence of External Crohn's Disease Risk Factors on Intestinal Bacteria. *Front Immunol*. 2015;6:558. doi:10.3389/fimmu.2015.00558.
20. Ricanek P, Lothe SM, Frye SA, Rydning A, Vatn MH, Tønjum T. Gut bacterial profile in

patients newly diagnosed with treatment-naïve Crohn's disease. *Clin Exp Gastroenterol*. 2012;5:173–86. doi:10.2147/CEG.S33858.

21. Liguori G, Lamas B, Richard ML, et al. Fungal Dysbiosis in Mucosa-associated Microbiota of Crohn's Disease Patients. *J Crohns Colitis*. 2015. doi:10.1093/ecco-jcc/jjv209.

22. Naftali T, Reshef L, Kovacs A, et al. Distinct Microbiotas are Associated with Ileum-Restricted and Colon-Involving Crohn's Disease. *Inflamm Bowel Dis*. 2016;22(2):293–302. doi:10.1097/MIB.0000000000000662.

23. Youmans BP, Ajami NJ, Jiang Z-D, et al. Characterization of the human gut microbiome during travelers' diarrhea. *Gut Microbes*. 2015;6(2):110–9. doi:10.1080/19490976.2015.1019693.

24. Quince C, Ijaz UZ, Loman N, et al. Extensive Modulation of the Fecal Metagenome in Children With Crohn's Disease During Exclusive Enteral Nutrition. *Am J Gastroenterol*. 2015;110(12):1718–29. doi:10.1038/ajg.2015.357.

25. Zaura E, Brandt BW, Teixeira de Mattos MJ, et al. Same Exposure but Two Radically Different Responses to Antibiotics: Resilience of the Salivary Microbiome versus Long-Term Microbial Shifts in Feces. *MBio*. 2015;6(6):e01693–15–e01693–15. doi:10.1128/mBio.01693-15.

26. Hashash JG, Chintamaneni P, Ramos Rivers CM, et al. Patterns of Antibiotic Exposure and Clinical Disease Activity in Inflammatory Bowel Disease: A 4-year Prospective Study. *Inflamm Bowel Dis*. 2015;21(11):2576–82. doi:10.1097/MIB.0000000000000534.

27. Lee T, Clavel T, Smirnov K, et al. Oral versus intravenous iron replacement therapy distinctly alters the gut microbiota and metabolome in patients with IBD. *Gut*. 2016. doi:10.1136/gutjnl-2015-309940.

28. Bashan A, Gibson TE, Friedman J, et al. Universality of human microbial dynamics. *Nature*. 2016;534(7606):259–62. doi:10.1038/nature18301.

29. Cosnes J, Gower-Rousseau C, Seksik P, Cortot A. Epidemiology and natural history of inflammatory bowel diseases. *Gastroenterology*. 2011;140(6):1785–94. doi:10.1053/j.gastro.2011.01.055.

30. Versini M, Jeandel P-Y, Bashi T, Bizzaro G, Blank M, Shoenfeld Y. Unraveling the Hygiene Hypothesis of helminthes and autoimmunity: origins, pathophysiology, and clinical applications. *BMC Med*. 2015;13:81. doi:10.1186/s12916-015-0306-7.

31. Black RE, Cousens S, Johnson HL, et al. Global, regional, and national causes of child mortality in 2008: a systematic analysis. *Lancet*. 2010;375(9730):1969–87. doi:10.1016/S0140-6736(10)60549-1.

32. Levine MM, Kaper JB, Black RE, Clements ML. New knowledge on pathogenesis of bacterial enteric infections as applied to vaccine development. *Microbiol Rev*. 1983;47(4):510–50. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=281589&tool=pmcentrez&rendertype=abstract. Accessed March 25, 2016.

33. Ahuja V, Tandon RK. Inflammatory bowel disease in the Asia-Pacific area: a comparison with developed countries and regional differences. *J Dig Dis*. 2010;11(3):134–47. doi:10.1111/j.1751-2980.2010.00429.x.

34. Victoria CR, Sassak LY, Nunes HR de C. Incidence and prevalence rates of inflammatory bowel diseases, in midwestern of São Paulo State, Brazil. *Arq Gastroenterol*. 2009;46(1):20–5. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19466305. Accessed February 27, 2015.

35. Hu D, Ren J, Wang G, et al. Geographic mapping of Crohn's disease and its relation to affluence in jiangsu province, an eastern coastal province of china. *Gastroenterol Res Pract*. 2014;2014:590467. doi:10.1155/2014/590467.

36. Mayberry J, Mann R. Inflammatory bowel disease in rural sub-Saharan Africa: rarity of diagnosis in patients attending mission hospitals. *Digestion*. 1989;44(3):172–6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/2628138. Accessed March 6, 2015.

37. Guerrant RL, DeBoer MD, Moore SR, Scharf RJ, Lima AAM. The impoverished gut--a triple burden of diarrhoea, stunting and chronic disease. *Nat Rev Gastroenterol Hepatol*. 2013;10(4):220–9. doi:10.1038/nrgastro.2012.239.

38. Lahariya C, Paul VK. Burden, differentials, and causes of child deaths in India. *Indian J Pediatr*. 2010;77(11):1312–21. doi:10.1007/s12098-010-0185-z.

39. Kirk MD, Pires SM, Black RE, et al. World Health Organization Estimates of the Global and Regional Disease Burden of 22 Foodborne Bacterial, Protozoal, and Viral Diseases, 2010: A Data Synthesis. *PLoS Med*. 2015;12(12):e1001921. doi:10.1371/journal.pmed.1001921.

40. Ojha SC, Jaide C, Jinawath N, Rotjanapan P, Baral P. Geohelminths: public health significance. *J Infect Dev Ctries*. 2014;8(1):5–16. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24423707. Accessed March 6, 2015.

41. Chu KM, Watermeyer G, Shelly L, et al. Childhood helminth exposure is protective against inflammatory bowel disease: a case control study in South Africa. *Inflamm Bowel Dis*. 2013;19(3):614–20. doi:10.1097/MIB.0b013e31827f27f4.

42. Bager P, Vinkel Hansen A, Wohlfahrt J, Melbye M. Helminth infection does not reduce risk for chronic inflammatory disease in a population-based cohort study. *Gastroenterology*. 2012;142(1):55–62. doi:10.1053/j.gastro.2011.09.046.

43. Garg SK, Croft AM, Bager P. Helminth therapy (worms) for induction of remission in inflammatory bowel disease. *Cochrane database Syst Rev*. 2014;1:CD009400. doi:10.1002/14651858.CD009400.pub2.

44. Nyachuba DG. Foodborne illness: is it on the rise? *Nutr Rev*. 2010;68(5):257–69. doi:10.1111/j.1753-4887.2010.00286.x.

45. Khabbaz RF, Moseley RR, Steiner RJ, Levitt AM, Bell BP. Challenges of infectious diseases in the USA. *Lancet (London, England)*. 2014;384(9937):53–63. doi:10.1016/S0140-6736(14)60890-4.

46. Gormley FJ, Little CL, Rawal N, Gillespie IA, Lebaigue S, Adak GK. A 17-year review of foodborne outbreaks: describing the continuing decline in England and Wales (1992-2008). *Epidemiol Infect*. 2011;139(5):688–99. doi:10.1017/S0950268810001858.

47. Kumagai Y, Gilmour S, Ota E, et al. Estimating the burden of foodborne diseases in Japan. *Bull World Health Organ*. 2015;93(8):540–549C. doi:10.2471/BLT.14.148056.

48. Butler AJ, Thomas MK, Pintar KDM. Expert elicitation as a means to attribute 28 enteric pathogens to foodborne, waterborne, animal contact, and person-to-person transmission routes in Canada. *Foodborne Pathog Dis*. 2015;12(4):335–44. doi:10.1089/fpd.2014.1856.

49. Sonnenberg A. Age distribution of IBD hospitalization. *Inflamm Bowel Dis*. 2010;16(3):452–7. doi:10.1002/ibd.21058.

50. Loftus E V, Schoenfeld P, Sandborn WJ. The epidemiology and natural history of Crohn's disease in population-based patient cohorts from North America: a systematic review. *Aliment Pharmacol Ther*. 2002;16(1):51–60. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11856078. Accessed February 10, 2016.

51. Burisch J, Pedersen N, Čuković-Čavka S, et al. East-West gradient in the incidence of inflammatory bowel disease in Europe: the ECCO-EpiCom inception cohort. *Gut*. 2014;63(4):588–97. doi:10.1136/gutjnl-2013-304636.

52. Murray CJL, Ortblad KF, Guinovart C, et al. Global, regional, and national incidence and mortality for HIV, tuberculosis, and malaria during 1990–2013: a systematic analysis for the Global Burden of Disease Study 2013. *Lancet*. 2014;384(9947):1005–70. doi:10.1016/S0140-6736(14)60844-8.

53. Hung C-C, Chang S-Y, Ji D-D. Entamoeba histolytica infection in men who have sex with men. *Lancet Infect Dis*. 2012;12(9):729–36. doi:10.1016/S1473-3099(12)70147-0.

54. Simms I, Field N, Jenkins C, et al. Intensified shigellosis epidemic associated with sexual transmission in men who have sex with men--Shigella flexneri and S. sonnei in England, 2004 to end of February 2015. *Euro Surveill Bull Eur sur les Mal Transm = Eur Commun*

Dis Bull. 2015;20(15). Available at: http://www.ncbi.nlm.nih.gov/pubmed/25953129. Accessed January 20, 2016.

55. Danila RN, Eikmeier DL, Robinson TJ, La Pointe A, DeVries AS. Two concurrent enteric disease outbreaks among men who have sex with men, minneapolis-st paul area. *Clin Infect Dis*. 2014;59(7):987–9. doi:10.1093/cid/ciu478.

56. Gaudreau C, Helferty M, Sylvestre J-L, et al. Campylobacter coli outbreak in men who have sex with men, Quebec, Canada, 2010-2011. *Emerg Infect Dis*. 2013;19(5):764–7. doi:10.3201/eid1905.121344.

57. Aragon TJ, Vugia DJ, Shallow S, et al. Case-Control Study of Shigellosis in San Francisco: The Role of Sexual Transmission and HIV Infection. *Clin Infect Dis*. 2007;44(3):327–334. doi:10.1086/510593.

58. Salit IE, Khairnar K, Gough K, Pillai DR. A possible cluster of sexually transmitted Entamoeba histolytica: genetic analysis of a highly virulent strain. *Clin Infect Dis*. 2009;49(3):346–53. doi:10.1086/600298.

59. Edwards S, Carne C. Oral sex and transmission of non-viral STIs. *Sex Transm Infect*. 1998;74(2):95–100. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1758102&tool=pmcentrez&rendertype=abstract. Accessed April 18, 2016.

60. Mercer CH, Tanton C, Prah P, et al. Changes in sexual attitudes and lifestyles in Britain through the life course and over time: findings from the National Surveys of Sexual Attitudes and Lifestyles (Natsal). *Lancet (London, England)*. 2013;382(9907):1781–94. doi:10.1016/S0140-6736(13)62035-8.

61. Benson LS, Martins SL, Whitaker AK. Correlates of Heterosexual Anal Intercourse among Women in the 2006-2010 National Survey of Family Growth. *J Sex Med*. 2015;12(8):1746–52. doi:10.1111/jsm.12961.

62. Halperin DT. Heterosexual anal intercourse: prevalence, cultural factors, and HIV infection and other health risks, Part I. *AIDS Patient Care STDS*. 1999;13(12):717–30. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10743535. Accessed February 9, 2016.

63. Cunningham SD, Olthoff G, Burnett P, Rompalo AM, Ellen JM. Evidence of heterosexual bridging among syphilis-positive men who have sex with men. *Sex Transm Infect*. 2006;82(6):444–5. doi:10.1136/sti.2005.019513.

64. Zheng JJ, Zhu XS, Huangfu Z, Shi XH, Guo ZR. Prevalence and incidence rates of Crohn's disease in mainland China: a meta-analysis of 55 years of research. *J Dig Dis*. 2010;11(3):161–6. doi:10.1111/j.1751-2980.2010.00431.x.

65. Al-Mofarreh MA, Al-Mofleh IA. Emerging inflammatory bowel disease in saudi outpatients: a report of 693 cases. *Saudi J Gastroenterol*. 19(1):16–22. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3603484&tool=pmcentrez&rendertype=abstract. Accessed March 24, 2016.

66. Ooi CJ, Makharia GK, Hilmi I, et al. Asia Pacific Consensus Statements on Crohn's disease. Part 1: Definition, diagnosis, and epidemiology: (Asia Pacific Crohn's Disease Consensus-Part 1). *J Gastroenterol Hepatol*. 2016;31(1):45–55. doi:10.1111/jgh.12956.

67. Ng JYS, Wong M-L, Chan RKW, Sen P, Chio MTW, Koh D. Gender Differences in Factors Associated With Anal Intercourse Among Heterosexual Adolescents in Singapore. *AIDS Educ Prev*. 2015;27(4):373–85. doi:10.1521/aeap.2015.27.4.373.

68. Segal AW, Loewi G. Neutrophil dysfunction in Crohn's disease. *Lancet*. 1976;2:219–221.

69. Marks DJ, Harbord MW, MacAllister R, et al. Defective acute inflammation in Crohn's disease: a clinical investigation. *Lancet*. 2006;367(9511):668–678.

70. Smith AM, Rahman FZ, Hayee B, et al. Disordered macrophage cytokine secretion underlies impaired acute inflammation and bacterial clearance in Crohn's disease. *JExpMed*. 2009;206(9):1883–1897.

71. Chew TS, O'Shea NR, Sewell GW, et al. Optineurin deficiency contributes to impaired

cytokine secretion and neutrophil recruitment in bacteria driven colitis. *Dis Model Mech*. 2015. doi:10.1242/dmm.020362.

72. Smith AM, Sewell GW, Levine AP, et al. Disruption of macrophage pro-inflammatory cytokine release in Crohn's disease is associated with reduced optineurin expression in a subset of patients. *Immunology*. 2015;144(1):45–55. doi:10.1111/imm.12338.

73. Levine AP, Pontikos N, Schiff ER, et al. Genetic Complexity of Crohn's Disease in 2 Large Ashkenazi Jewish Families. *Gastroenterology*. 2016. doi:10.1053/j.gastro.2016.06.040.

74. De Bruin AFJ, Schouten SB, de Kort PPS, Gosselink MP, van der Harst E. The impact of chronic smoking on rectal mucosal blood flow. *Tech Coloproctol*. 2009;13(4):269–72. doi:10.1007/s10151-009-0529-8.

75. Segal AW, Arnot RN, Thakur ML, Lavender JP. Indium-111-labelled leucocytes for localisation of abscesses. *Lancet*. 1976;2:1056–1058.

76. Elliott TR, Hudspith BN, Rayment NB, et al. Defective macrophage handling of Escherichia coli in Crohn's disease. *J Gastroenterol Hepatol*. 2015;30(8):1265–74. doi:10.1111/jgh.12955.

77. Campos N, Magro F, Castro AR, et al. Macrophages from IBD patients exhibit defective tumour necrosis factor-α secretion but otherwise normal or augmented pro-inflammatory responses to infection. *Immunobiology*. 2011;216(8):961–70. doi:10.1016/j.imbio.2011.01.002.

78. Sewell GW, Rahman FZ, Levine AP, et al. Defective tumor necrosis factor release from Crohn's disease macrophages in response to Toll-like receptor activation: relationship to phenotype and genome-wide association susceptibility loci. *Inflamm Bowel Dis*. 2012;18(11):2120–7. doi:10.1002/ibd.22952.

79. Vazeille E, Buisson A, Bringer M-A, et al. Monocyte-derived macrophages from Crohn's disease patients are impaired in the ability to control intracellular adherent-invasive Escherichia coli and exhibit disordered cytokine secretion profile. *J Crohns Colitis*. 2015;9(5):410–20. doi:10.1093/ecco-jcc/jjv053.

80. Levine AP, Segal AW. What is wrong with granulocytes in inflammatory bowel diseases? *Dig Dis*. 2013;31(3-4):321–7. doi:10.1159/000354686.

81. Wandall JH, Binder V. Leucocyte function in Crohn's disease. Studies on mobilisation using a quantitative skin window technique and on the function of circulating polymorphonuclear leucocytes in vitro. *Gut*. 1982;23(3):173–80. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1419627&tool=pmcentrez&rendertype=abstract. Accessed March 1, 2016.

82. Somasundaram R, Nuij VJAA, van der Woude CJ, Kuipers EJ, Peppelenbosch MP, Fuhler GM. Peripheral neutrophil functions and cell signalling in Crohn`s disease. *PLoS One*. 2013;8(12):e84521. doi:10.1371/journal.pone.0084521.

83. Marks DJB, Miyagi K, Rahman FZ, Novelli M, Bloom SL, Segal AW. Inflammatory bowel disease in CGD reproduces the clinicopathological features of Crohn's disease. *Am J Gastroenterol*. 2009;104(1):117–24. doi:10.1038/ajg.2008.72.

84. Ahmad T, Satsangi J, McGovern D, Bunce M, Jewell DP. Review article: the genetics of inflammatory bowel disease. *Aliment Pharmacol Ther*. 2001;15(6):731–48. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11380312. Accessed March 1, 2016.

85. Moller FT, Andersen V, Wohlfahrt J, Jess T. Familial risk of inflammatory bowel disease: a population-based cohort study 1977-2011. *Am J Gastroenterol*. 2015;110(4):564–71. doi:10.1038/ajg.2015.50.

86. Brant SR. Update on the heritability of inflammatory bowel disease: the importance of twin studies. *Inflamm Bowel Dis*. 2011;17(1):1–5. doi:10.1002/ibd.21385.

87. Hugot JP, Chamaillard M, Zouali H, et al. Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature*. 2001;411(6837):599–603. doi:10.1038/35079107.

88. Ogura Y, Bonen DK, Inohara N, et al. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature*. 2001;411(6837):603–6. doi:10.1038/35079114.

89. Risch N, Merikangas K. The future of genetic studies of complex human diseases. *Science*. 1996;273(5281):1516–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8801636. Accessed March 15, 2015.

90. Pe'er I, Yelensky R, Altshuler D, Daly MJ. Estimation of the multiple testing burden for genomewide association studies of nearly all common variants. *Genet Epidemiol*. 2008;32(4):381–5. doi:10.1002/gepi.20303.

91. Spencer CCA, Su Z, Donnelly P, Marchini J. Designing Genome-Wide Association Studies: Sample Size, Power, Imputation, and the Choice of Genotyping Chip. Storey JD, ed. *PLoS Genet*. 2009;5(5):e1000477. doi:10.1371/journal.pgen.1000477.

92. Hirschhorn JN, Daly MJ. Genome-wide association studies for common diseases and complex traits. *Nat Rev Genet*. 2005;6(2):95–108. doi:10.1038/nrg1521.

93. Jostins L, Ripke S, Weersma RK, et al. Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. *Nature*. 2012;491(7422):119–24. doi:10.1038/nature11582.

94. Ellinghaus D, Jostins L, Spain SL, et al. Analysis of five chronic inflammatory diseases identifies 27 new associations and highlights disease-specific patterns at shared loci. *Nat Genet*. 2016. doi:10.1038/ng.3528.

95. de Lange KM, Moutsianas L, Lee JC, et al. Genome--wide association study implicates immune activation of multiple integrin genes in inflammatory bowel disease 2. doi:10.1101/058255.

96. Liu JZ, van Sommeren S, Huang H, et al. Association analyses identify 38 susceptibility loci for inflammatory bowel disease and highlight shared genetic risk across populations. *Nat Genet*. 2015;47(9):979–86. doi:10.1038/ng.3359.

97. Brown MA, Pile KD, Kennedy LG, et al. HLA class I associations of ankylosing spondylitis in the white population in the United Kingdom. *Ann Rheum Dis*. 1996;55(4):268–70. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1010149&tool=pmcentrez&rendertype=abstract. Accessed June 2, 2016.

98. Chinn S. A simple method for converting an odds ratio to effect size for use in meta-analysis. *Stat Med*. 2000;19(22):3127–31. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11113947. Accessed June 2, 2016.

99. Smigoc Schweiger D, Mendez A, Kunilo Jamnik S, et al. High-risk genotypes HLA-DR3-DQ2/DR3-DQ2 and DR3-DQ2/DR4-DQ8 in co-occurrence of type 1 diabetes and celiac disease. *Autoimmunity*. 2016;49(4):240–7. doi:10.3109/08916934.2016.1164144.

100. Zhernakova A, van Diemen CC, Wijmenga C. Detecting shared pathogenesis from the shared genetics of immune-related diseases. *Nat Rev Genet*. 2009;10(1):43–55. doi:10.1038/nrg2489.

101. Slowicka K, Vereecke L, McGuire C, et al. Optineurin deficiency in mice is associated with increased sensitivity to Salmonella but does not affect proinflammatory NF-κB signaling. *Eur J Immunol*. 2015. doi:10.1002/eji.201545863.

102. Ying H, Yue BYJT. Cellular and molecular biology of optineurin. *Int Rev Cell Mol Biol*. 2012;294:223–58. doi:10.1016/B978-0-12-394305-7.00005-7.

103. Wild P, Farhan H, McEwan DG, et al. Phosphorylation of the autophagy receptor optineurin restricts Salmonella growth. *Science*. 2011;333(6039):228–33. doi:10.1126/science.1205405.

104. Sawyer SL, Hartley T, Dyment DA, et al. Utility of whole-exome sequencing for those near the end of the diagnostic odyssey: time to address gaps in care. *Clin Genet*. 2016;89(3):275–84. doi:10.1111/cge.12654.

105. Stranneheim H, Wedell A. Exome and genome sequencing: a revolution for the discovery

and diagnosis of monogenic disorders. *J Intern Med*. 2016;279(1):3–15. doi:10.1111/joim.12399.

106. Prescott NJ, Lehne B, Stone K, et al. Pooled sequencing of 531 genes in inflammatory bowel disease identifies an associated rare variant in BTNL2 and implicates other immune related genes. *PLoS Genet*. 2015;11(2):e1004955. doi:10.1371/journal.pgen.1004955.

107. Cardinale CJ, Kelsen JR, Baldassano RN, Hakonarson H. Impact of exome sequencing in inflammatory bowel disease. *World J Gastroenterol*. 2013;19(40):6721–9. doi:10.3748/wjg.v19.i40.6721.

108. Uhlig HH, Schwerd T, Koletzko S, et al. The diagnostic approach to monogenic very early onset inflammatory bowel disease. *Gastroenterology*. 2014;147(5):990–1007.e3. doi:10.1053/j.gastro.2014.07.023.

109. MacArthur DG, Balasubramanian S, Frankish A, et al. A systematic survey of loss-of-function variants in human protein-coding genes. *Science*. 2012;335(6070):823–8. doi:10.1126/science.1215040.

110. Luo Y, de Lange KM, Jostins L, et al. *Exploring the genetic architecture of inflammatory bowel disease by whole genome sequencing identifies association at ADCY7*. Cold Spring Harbor Labs Journals; 2016. doi:10.1101/058347.

111. Carmi S, Hui KY, Kochav E, et al. Sequencing an Ashkenazi reference panel supports population-targeted personal genomics and illuminates Jewish and European origins. *Nat Commun*. 2014;5:4835. doi:10.1038/ncomms5835.

112. Chuang L-S, Villaverde N, Hui KY, et al. A Frameshift in CSF2RB Predominant Among Ashkenazi Jews Increases Risk for Crohn's Disease and Reduces Monocyte Signaling via GMCSF. *Gastroenterology*. 2016. doi:10.1053/j.gastro.2016.06.045.

113. Zhang W, Hui KY, Gusev A, et al. Extended haplotype association study in Crohn's disease identifies a novel, Ashkenazi Jewish-specific missense mutation in the NF-κB pathway gene, HEATR3. *Genes Immun*. 2013;14(5):310–6. doi:10.1038/gene.2013.19.

114. Gardet A, Benita Y, Li C, et al. LRRK2 is involved in the IFN-gamma response and host response to pathogens. *J Immunol*. 2010;185(9):5577–85. doi:10.4049/jimmunol.1000548.

115. Sekhwal MK, Li P, Lam I, Wang X, Cloutier S, You FM. Disease Resistance Gene Analogs (RGAs) in Plants. *Int J Mol Sci*. 2015;16(8):19248–90. doi:10.3390/ijms160819248.

116. Gay NJ, Symmons MF, Gangloff M, Bryant CE. Assembly and localization of Toll-like receptor signalling complexes. *Nat Rev Immunol*. 2014;14(8):546–558. doi:10.1038/nri3713.

117. Nakamura N, Lill JR, Phung Q, et al. Endosomes are specialized platforms for bacterial sensing and NOD2 signalling. *Nature*. 2014;509(7499):240–4. doi:10.1038/nature13133.

118. Keestra-Gounder AM, Byndloss MX, Seyffert N, et al. NOD1 and NOD2 signalling links ER stress with inflammation. *Nature*. 2016;532(7599):394–7. doi:10.1038/nature17631.

119. Broughton SE, Dhagat U, Hercus TR, et al. The GM-CSF/IL-3/IL-5 cytokine receptor family: from ligand recognition to initiation of signaling. *Immunol Rev*. 2012;250(1):277–302. doi:10.1111/j.1600-065X.2012.01164.x.

120. Wicks IP, Roberts AW. Targeting GM-CSF in inflammatory diseases. *Nat Rev Rheumatol*. 2015;12(1):37–48. doi:10.1038/nrrheum.2015.161.

121. Metcalf D. Hematopoietic cytokines. *Blood*. 2008;111(2):485–91. doi:10.1182/blood-2007-03-079681.

122. Sanderson CJ. Eosinophil differentiation factor (interleukin-5). *Immunol Ser*. 1990;49:231–56. Available at: http://www.ncbi.nlm.nih.gov/pubmed/2090253. Accessed March 16, 2016.

123. Tschopp J, Martinon F, Burns K. NALPs: a novel protein family involved in inflammation. *Nat Rev Mol Cell Biol*. 2003;4(2):95–104. doi:10.1038/nrm1019.

124. Sharma D, Kanneganti T-D. The cell biology of inflammasomes: Mechanisms of inflammasome activation and regulation. *J Cell Biol*. 2016;213(6):617–29. doi:10.1083/jcb.201602089.

125. Schapansky J, Nardozzi JD, LaVoie MJ. The complex relationships between microglia, alpha-synuclein, and LRRK2 in Parkinson's disease. *Neuroscience*. 2015;302:74–88. doi:10.1016/j.neuroscience.2014.09.049.

126. Waschbüsch D, Michels H, Strassheim S, et al. LRRK2 transport is regulated by its novel interacting partner Rab32. *PLoS One*. 2014;9(10):e111632. doi:10.1371/journal.pone.0111632.

127. Spanò S. Host restriction in Salmonella: insights from Rab GTPases. *Cell Microbiol*. 2014;16(9):1321–8. doi:10.1111/cmi.12327.

128. Hoffmann C, Finsel I, Otto A, et al. Functional analysis of novel Rab GTPases identified in the proteome of purified Legionella-containing vacuoles from macrophages. *Cell Microbiol*. 2014;16(7):1034–52. doi:10.1111/cmi.12256.

129. Gerondopoulos A, Langemeyer L, Liang J-R, Linford A, Barr FA. BLOC-3 mutated in Hermansky-Pudlak syndrome is a Rab32/38 guanine nucleotide exchange factor. *Curr Biol*. 2012;22(22):2135–9. doi:10.1016/j.cub.2012.09.020.

130. Lin J-C, Lin C-S, Hsu C-W, Lin C-L, Kao C-H. Association Between Parkinson's Disease and Inflammatory Bowel Disease: a Nationwide Taiwanese Retrospective Cohort Study. *Inflamm Bowel Dis*. 2016. doi:10.1097/MIB.0000000000000735.

131. Nalls MA, Saad M, Noyce AJ, et al. Genetic comorbidities in Parkinson's disease. *Hum Mol Genet*. 2014;23(3):831–41. doi:10.1093/hmg/ddt465.

132. Kouklakis G, Efremidou EI, Papageorgiou MS, Pavlidou E, Manolas KJ, Liratzopoulos N. Complicated Crohn's-like colitis, associated with Hermansky-Pudlak syndrome, treated with Infliximab: a case report and brief review of the literature. *J Med Case Rep*. 2007;1:176. doi:10.1186/1752-1947-1-176.

133. Tumbarello DA, Kendrick-Jones J, Buss F. Myosin VI and its cargo adaptors - linking endocytosis and autophagy. *J Cell Sci*. 2013;126(Pt 12):2561–70. doi:10.1242/jcs.095554.

134. Ying H, Yue BYJT. Optineurin: The autophagy connection. *Exp Eye Res*. 2016;144:73–80. doi:10.1016/j.exer.2015.06.029.

135. Bedard K, Krause KH. The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. *Physiol Rev*. 2007;87(1):245–313.

136. Segal AW. NADPH oxidases as electrochemical generators to produce ion fluxes and turgor in fungi, plants and humans. *Open Biol*. 2016;6(5). doi:10.1098/rsob.160028.

137. Segal AW, Jones OTG. Novel cytochrome b system in phagocytic vacuoles from human granulocytes. *Nature*. 1978;276:515–517.

138. Sommer F, Bäckhed F. The gut microbiota engages different signaling pathways to induce Duox2 expression in the ileum and colon epithelium. *Mucosal Immunol*. 2014. doi:10.1038/mi.2014.74.

139. Rada B, Leto TL. Oxidative innate immune defenses by Nox/Duox family NADPH oxidases. *Contrib Microbiol*. 2008;15:164–87. doi:10.1159/000136357.

140. Chang S, Linderholm A, Franzi L, Kenyon N, Grasberger H, Harper R. Dual oxidase regulates neutrophil recruitment in allergic airways. *Free Radic Biol Med*. 2013;65:38–46. doi:10.1016/j.freeradbiomed.2013.06.012.

141. Grasberger H, El-Zaatari M, Dang DT, Merchant JL. Dual oxidases control release of hydrogen peroxide by the gastric epithelium to prevent Helicobacter felis infection and inflammation in mice. *Gastroenterology*. 2013;145(5):1045–54. doi:10.1053/j.gastro.2013.07.011.

142. Lipinski S, Till A, Sina C, et al. DUOX2-derived reactive oxygen species are effectors of NOD2-mediated antibacterial responses. *J Cell Sci*. 2009;122(Pt 19):3522–30. doi:10.1242/jcs.050690.

143. Broeze KA, Opmeer BC, Coppus SFPJ, et al. Chlamydia antibody testing and diagnosing tubal pathology in subfertile women: an individual patient data meta-analysis. *Hum Reprod Update*. 2011;17(3):301–10. doi:10.1093/humupd/dmq060.

144. Yarbrough ML, Burnham C-AD. The ABCs of STIs: An Update on Sexually Transmitted Infections. *Clin Chem*. 2016;62(6):811–23. doi:10.1373/clinchem.2015.240234.

145. Hindryckx P, Baert F, Hart A, Armuzzi A, Panès J, Peyrin-Biroulet L. Clinical trials in luminal Crohn's disease: A historical perspective. *J Crohns Colitis*. 2014. doi:10.1016/j.crohns.2014.04.007.

146. Hedin CR, Stagg AJ, Whelan K, Lindsay JO. Family studies in Crohn's disease: new horizons in understanding disease pathogenesis, risk and prevention. *Gut*. 2012;61(2):311–8. doi:10.1136/gut.2011.238568.

147. Geiszt M, Witta J, Baffi J, Lekstrom K, Leto TL. Dual oxidases represent novel hydrogen peroxide sources supporting mucosal surface host defense. *FASEB J*. 2003;17(11):1502–4. doi:10.1096/fj.02-1104fje.

148. Johansson ME V, Gustafsson JK, Holmén-Larsson J, et al. Bacteria penetrate the normally impenetrable inner colon mucus layer in both murine colitis models and patients with ulcerative colitis. *Gut*. 2014;63(2):281–91. doi:10.1136/gutjnl-2012-303207.

149. Johansson ME V, Phillipson M, Petersson J, Velcich A, Holm L, Hansson GC. The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria. *Proc Natl Acad Sci U S A*. 2008;105(39):15064–9. doi:10.1073/pnas.0803124105.

150. Chu H, Khosravi A, Kusumawardhani IP, et al. Gene-microbiota interactions contribute to the pathogenesis of inflammatory bowel disease. *Science*. 2016;352(6289):1116–20. doi:10.1126/science.aad9948.

151. Al Nabhani Z, Lepage P, Mauny P, et al. Nod2 Deficiency Leads to a Specific and Transmissible Mucosa-associated Microbial Dysbiosis Which Is Independent of the Mucosal Barrier Defect. *J Crohns Colitis*. 2016. doi:10.1093/ecco-jcc/jjw095.

152. Cox MJ, Cookson WOCM, Moffatt MF. Sequencing the human microbiome in health and disease. *Hum Mol Genet*. 2013;22(R1):R88–94. doi:10.1093/hmg/ddt398.

153. O'Shea NR, Chew TS, Dunne J, et al. Critical role of the disintegrin metalloprotease ADAM-like Decysin-1 (ADAMDEC1) for intestinal immunity and inflammation. *J Crohns Colitis*. 2016. doi:10.1093/ecco-jcc/jjw111.

154. Schulthess J, Meresse B, Ramiro-Puig E, Montcuquet N, Darche S, Bègue B, Ruemmele F, Combadière C, Di Santo JP, Buzoni-Gatel D C-BN. Interleukin-15-dependent NKp46+ innate lymphoid cells control intestinal inflammation by recruiting inflammatory monocytes. *Immunity*. 2012;37(1):108–121.

155. Marks J, Srai SK, Biber J, Murer H, Unwin RJ, Debnam ES. Intestinal phosphate absorption and the effect of vitamin D: a comparison of rats with mice. *Exp Physiol*. 2006;91(3):531–7. doi:10.1113/expphysiol.2005.032516.

156. Maekawa S, Ohto U, Shibata T, Miyake K, Shimizu T. Crystal structure of NOD2 and its implications in human disease. *Nat Commun*. 2016;7:11813. doi:10.1038/ncomms11813.

157. Jun JC, Cominelli F, Abbott DW. RIP2 activity in inflammatory disease and implications for novel therapeutics. *J Leukoc Biol*. 2013;94(5):927–32. doi:10.1189/jlb.0213109.

158. Petnicki-Ocwieja T, Kern A. Mechanisms of Borrelia burgdorferi internalization and intracellular innate immune signaling. *Front Cell Infect Microbiol*. 2014;4:175. doi:10.3389/fcimb.2014.00175.

159. Mizushima N, Noda T, Yoshimori T, et al. A protein conjugation system essential for autophagy. *Nature*. 1998;395(6700):395–8. doi:10.1038/26506.

160. Narayanan LA, Edelmann MJ. Ubiquitination as an efficient molecular strategy employed in salmonella infection. *Front Immunol*. 2014;5:558. doi:10.3389/fimmu.2014.00558.

161. Kawabata T, Yoshimori T. Beyond starvation: An update on the autophagic machinery and its functions. *J Mol Cell Cardiol*. 2015. doi:10.1016/j.yjmcc.2015.12.005.

162. Sorbara MT, Girardin SE. Emerging themes in bacterial autophagy. *Curr Opin Microbiol*. 2015;23:163–70. doi:10.1016/j.mib.2014.11.020.

163. Kimmey JM, Huynh JP, Weiss LA, et al. Unique role for ATG5 in neutrophil-mediated immunopathology during M. tuberculosis infection. *Nature*. 2015;528(7583):565–9. doi:10.1038/nature16451.

164. Chanput W, Mes JJ, Wichers HJ. THP-1 cell line: an in vitro cell model for immune modulation approach. *Int Immunopharmacol*. 2014;23(1):37–45. doi:10.1016/j.intimp.2014.08.002.

165. Wang GG, Calvo KR, Pasillas MP, Sykes DB, Häcker H, Kamps MP. Quantitative production of macrophages or neutrophils ex vivo using conditional Hoxb8. *Nat Methods*. 2006;3(4):287–93. doi:10.1038/nmeth865.

166. Schmidt F, Grimm D. CRISPR genome engineering and viral gene delivery: a case of mutual attraction. *Biotechnol J*. 2015;10(2):258–72. doi:10.1002/biot.201400529.

167. Wang X, Kuivaniemi H, Bonavita G, et al. CARD15 mutations in familial granulomatosis syndromes: a study of the original Blau syndrome kindred and other families with large-vessel arteritis and cranial neuropathy. *Arthritis Rheum*. 2002;46(11):3041–5. doi:10.1002/art.10618.

168. Chen X, Wei S, Ji Y, Guo X, Yang F. Quantitative proteomics using SILAC: Principles, applications, and developments. *Proteomics*. 2015;15(18):3175–92. doi:10.1002/pmic.201500108.

169. Gross O. Measuring the inflammasome. *Methods Mol Biol*. 2012;844:199–222. doi:10.1007/978-1-61779-527-5_15.

170. Stutz A, Horvath GL, Monks BG, Latz E. ASC speck formation as a readout for inflammasome activation. *Methods Mol Biol*. 2013;1040:91–101. doi:10.1007/978-1-62703-523-1_8.

171. Beilharz M, De Nardo's D, Latz E, Franklin BS. Measuring NLR Oligomerization II: Detection of ASC Speck Formation by Confocal Microscopy and Immunofluorescence. *Methods Mol Biol*. 2016;1417:145–58. doi:10.1007/978-1-4939-3566-6_9.

172. Dzamko N, Inesta-Vaquera F, Zhang J, et al. The IkappaB kinase family phosphorylates the Parkinson's disease kinase LRRK2 at Ser935 and Ser910 during Toll-like receptor signaling. *PLoS One*. 2012;7(6):e39132. doi:10.1371/journal.pone.0039132.

173. McCall P, Bennett L, Ahmad I, et al. NFκB signalling is upregulated in a subset of castrate-resistant prostate cancer patients and correlates with disease progression. *Br J Cancer*. 2012;107(9):1554–63. doi:10.1038/bjc.2012.372.

174. Varnaitė R, MacNeill SA. Meet the neighbours: mapping local protein interactomes by proximity-dependent labelling with BioID. *Proteomics*. 2016. doi:10.1002/pmic.201600123.

175. Kovalenko EI, Ranjbar S, Jasenosky LD, Goldfeld AE, Vorobjev IA, Barteneva NS. The use of HaloTag-based technology in flow and laser scanning cytometry analysis of live and fixed cells. *BMC Res Notes*. 2011;4:340. doi:10.1186/1756-0500-4-340.

176. Lopez-Cubero SO, Sullivan KM, McDonald GB. Course of Crohn's disease after allogeneic marrow transplantation. *Gastroenterology*. 1998;114(3):433–40. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9496932. Accessed November 3, 2014.

177. Yona S, Kim K-W, Wolf Y, et al. Fate mapping reveals origins and dynamics of monocytes and tissue macrophages under homeostasis. *Immunity*. 2013;38(1):79–91. doi:10.1016/j.immuni.2012.12.001.

178. Suzuki T, Arumugam P, Sakagami T, et al. Pulmonary macrophage transplantation therapy. *Nature*. 2014. doi:10.1038/nature13807.

Section c. Resources (including project costs)

Cost Category			Total in euro
Direct Costs [2]	**Personnel**	PI[3]	354,009.31
		Senior Staff	
		Postdocs (2 for 5 years, one for 3 years)	1131964.11
		Students	
		Other	
	i. Total Direct costs for Personnel (in euro)		1,485,973.41
	Travel		14,285
	Equipment		0
	Other goods and services	Consumables	216,953.44
		Audit	6,136.84
		Other (please specify) Animals	257,130.00
	ii. Total Other Direct Costs (in euro)		508,790.27
A – Total Direct Costs (i + ii) (in euro)			1,994,763.69
B – Indirect Costs (overheads) 25% of Direct Costs[4] (in euro)			498,690.92
C1 – Subcontracting Costs (no overheads) (in euro)			0
C2 – Other Direct Costs with no overheads[5] (in euro)			0
Total Estimated Eligible Costs (A + B + C) (in euro)[6]			2,493,454.61
Total Requested Grant (in euro)[6]			2,493,454.61

Request for additional funding above EUR 2 500 000 for	Justification
Keep only that category(ies) that apply to the project. (a) covering eligible 'start-up' costs for a PI moving from another country to the EU or an Associated Country as a consequence of receiving an ERC	

[2] An additional cost category 'Direct costing for Large Research Infrastructures' applicable to H2020 can be added to this table (below 'Other Goods and services') for PIs who are hosted by institutions with Large Research Infrastructures of a value of at least EUR 20 million and **only** after having received a positive ex-ante assessment from the Commission's services (see '*Information for Applicants to the Advanced Grant 2016 Call'* for more details).

[3] When calculating the salary, please take into account the percentage of your dedicated working time to run the ERC funded project (i.e. minimum 30% of your total working time).

[4] Please note that the overheads are fixed to a flat rate of exactly 25%.

[5] Such as the costs of resources made available by third parties which are not used on the premises of the beneficiary (see '*Information for Applicants to the Advanced Grant 2016 Call'* for details).

[6] These figures MUST match those presented in the online proposal submission form, section 3 – Budget.

grant and/or, (b) the purchase of major equipment and/or, (c) access to large facilities.	

Please indicate the duration of the project in months:[7]	**60**
Please indicate the % of working time the PI dedicates to the project over the period of the grant:	**30%**
Please indicate the % of working time the PI spends in an EU Member State or Associated Country over the period of the grant:	**100%**

Justification: All costs are in Euros, using UCL's official exchange rate of £1= €1.3333. The time required for the proposed experiments is based on previous experience of managing this sort of project, taking into account progress we have already made (e.g. mice part-generated at time of writing). **Aim 1:** 4 person-years, **Aim 2:** 3 person-years, **Aim 3:** 4 person-years, **Aim 4:** 4 person-years, **Total:** 15 person-years. On this basis I have requested 2 full-time postdocs (10 person-years), the gap to be filled by two PhD students (5 person-years, allowing for training).

Nature of the team:

Staff costs (total €1,485,973.41):

The salaries of the following personnel (1-4) will be fully or partly funded by the ERC-AdG:

1) Professor Anthony W Segal will lead the team. AWS will be closely involved with project design and will supervise other team members. He will spend 30% of his time on this project and his salary level is set at UCL Professor level 4.

2) Dr Francesca Semplici is a postdoctoral scientist and my laboratory manager. She will be responsible for the import and export of mice, all the breeding and maintenance of the mouse lines and will assist with the animal experiments.

3) I will require a **postdoctoral scientist to conduct the epidemiological studies – 3 years**. Interrogating the National Health Service Clinical Practice Research Datalink will take one year. The design and implementation of the sexual practises questionnaire will take a year as will the collection of blood samples and tests for evidence of prior STIs.

4) I will require **one postdoctoral scientist to undertake the animal experiments**. Together with Dr Semplici they will perform the bowel inflammation models, histology, FACS analysis, immunology, signalling pathway analysis, proteomics, biochemistry and cell biology of models with enhanced bowel inflammation.

The following personnel will work on the project but will be paid from other sources:

5) Postdoctoral geneticist. Dr Elena Schiff is funded by the Charles Wolfson Charitable Trust and is curating the smaller AJ families and sporadic cases with CD. She will assist with the further interaction and studies on the large AJ families and the collaboration with the Broad Institute.

[7] The maximum award is reduced pro rata temporis for projects of a shorter duration (e.g. for a project of 48 months duration the maximum requested EU contribution allowed is EUR 2 million). Additional funding to cover major one-off costs is not subject to pro-rata temporis reduction for projects of shorter duration (e.g. with additional funding it is possible to request a maximum EU contribution of EUR 3 million for a project of 48 months duration).

6) Two Clinical Fellows to be appointed. These two clinicians will be involved in the collaboration with the Hadassah Hospital. They will be funded by the Charles Wolfson Charitable trust to collect samples from AJ and Druze CD families in Israel for one year and then to come to UCL to prepare the DNA which will then be sequenced. They will then analyse the results and write the paper here over the second year. The results of these exome sequencing studies and those conducted by Dr Schiff will be integrated into the activation pathways and vesicle trafficking interacting proteins to cross validate candidate variants and functional protein networks.

7) **Bioinformatician** Mathew Frampton is a postdoctoral scientist funded from the Charles Wolfson Charitable Trust. He will assist with the exome sequence variant, and the pathway analysis.

Equipment cost: I have a well-founded laboratory and we do not require additional equipment.

Consumables (total €216,953.44): The cost of consumables is based on our experience and UCL guidelines. A total of €171,863 is needed to cover molecular biology reagents, commercial antibodies, bacterial culture media, disposable laboratoryware, DNA electrophoresis reagents, DNA sequencing (excluding exome sequencing), computer program licenses and upgrades, FACS and confocal microscopy. The licence to interrogate the National Health Service Clinical Practice Research Datalink is €20,000. The 3 ELISAs to test for the STIs each cost €470 for 96 tests. Thus 2,000 of each will be €29,375.

Animals: (total €257,130): The number of animals proposed here is based on our experience and advice from managers of our animal facility. We will initially study mouse 4 lines (*DUOX2 P303R, CSF2RB2 KO CSF2RB Ser709LEUfsTER22, HEATR3 Arg643Ser, NLRP2 Glu22Gly*) produced in Manchester at a cost of €43606.4. The import of LRRK2 KO and activating mutations from Dundee, and NOD2 KO mice from Lille will cost €1500. Each of the 6 lines in the 5 bowel inflammation models will require 435 mice (including breeders and 20% contingency) for an average of 11 weeks. We have also made allowance for the *DUOX2* mice to be bred on the NOD2 background. Out requirement for the conduct of these experiments is estimated to be €212023.6. Mouse costs will vary as the project develops but for accounting purposes are distributed equally over the five years of the project, including an amount for inflation as per UCL guidelines.

Publications: I have not requested funds for publication. In general I try to publish in journals that do not require payment for open access. If necessary where it is important to disseminate the information rapidly, we will publish online in a site like bioRxiv before subsequent publication in a journal as we have done previously.

Travel: I have requested funding for the postdoctoral scientists for one European conference (ie ECCO, totalling €4,285) and one inter-continental conferences (i.e. American Gastroenterological Association, Gordon Conference, totalling €10,000) each. The total cost of travel and overseas subsistence is €14,285.

Audit Costs: a total of €6,136.84 is specified by UCL for auditing. Audit costs are based on a Subcontract signed by UCL and ContractAuditline Limited (in line with UCL and national procurement procedures) which charges UCL per project audit.

Indirect costs: 25% of the Direct Costs listed above is requested.

Section 2D. Ethical issues table

	Research on Human Embryo/ Foetus	YES	Page
	Does the proposed research involve human Embryos?		
	Does the proposed research involve human Foetal Tissues/ Cells?		
	Does the proposed research involve human Embryonic Stem Cells (hESCs)?		
	Does the proposed research on human Embryonic Stem Cells involve cells in culture?		
	Does the proposed research on Human Embryonic Stem Cells involve the derivation of cells from Embryos?		
	I CONFIRM THAT NONE OF THE ABOVE ISSUES APPLY TO MY PROPOSAL	X	

	Research on Humans	YES	Page
	Does the proposed research involve children?		
	Does the proposed research involve patients?	X	10,11,12,15
	Does the proposed research involve persons not able to give consent?		
	Does the proposed research involve adult healthy volunteers?	X	10,11,12,15
	Does the proposed research involve Human genetic material?	X	10,11,12,15
	Does the proposed research involve Human biological samples?	X	10,11,12,15
	Does the proposed research involve Human data collection?	X	10,11,12,15
	I CONFIRM THAT NONE OF THE ABOVE ISSUES APPLY TO MY PROPOSAL		

	Privacy	YES	Page
	Does the proposed research involve processing of genetic information or personal data (e.g. health, sexual lifestyle, ethnicity, political opinion, religious or philosophical conviction)?	X	10,11
	Does the proposed research involve tracking the location or observation of people?		
	I CONFIRM THAT NONE OF THE ABOVE ISSUES APPLY TO MY PROPOSAL		

	Research on Animals	YES	Page
	Does the proposed research involve research on animals?	X	12,13
	Are those animals transgenic small laboratory animals?	X	12,13
	Are those animals transgenic farm animals?		
	Are those animals non-human primates?		
	Are those animals cloned farm animals?		
	I CONFIRM THAT NONE OF THE ABOVE ISSUES APPLY TO MY PROPOSAL		

	Research Involving non-EU Countries (ICPC Countries)	YES	Page
	Is the proposed research (or parts of it) going to take place in one or more of the ICPC Countries?		
	Is any material used in the research (e.g. personal data, animal and/or human tissue samples, genetic material, live animals, etc) : a) Collected in any of the ICPC countries?		
	b) Exported to any other country (including ICPC and EU Member States)?		
	I CONFIRM THAT NONE OF THE ABOVE ISSUES APPLY TO MY PROPOSAL	X	

	Dual Use	YES	Page
	Research having direct military use		
	Research having the potential for terrorist abuse		
	I CONFIRM THAT NONE OF THE ABOVE ISSUES APPLY TO MY PROPOSAL	X	

Section 2E. Time-line of research

Key intermediate goal or workpackage completion		estimated % of total grant	expected
Aim 1	Evidence of sexual transmission of CD	20	Dec 2020
Aim 2a	Further characterisation large AJ families	10	Dec 2020
Aim 3a	Generation of mutant mice	20	Dec 2018
Aim 3b	Testing mice for gastrointestinal infection	20	Dec 2019
Aim 3c	Generation and analysis cell based systems	10	Dec 2022
Aim 3d	Protein/protein interactions	15	Dec 2022
Aim 4	Investigation potential future treatments	5	Dec 2022

Section 3. The Research Environment

Host Institution: The work will be carried out in Rayne Institute at University College London (UCL). UCL is a large multi-faculty university in the centre of London. It is regularly rated in the top four research institutions in the National UK league tables and in the top twenty in International tables (e.g. ranked #7 in the QS World Rankings 2015/16; #18 in Shanghai Jiao Tong University Rankings 2015). UCL is a major hub for science in Europe, being close to the Eurostar terminal at King's Cross-St Pancras and the Francis Crick Institute, of which UCL is a founding partner along with the Wellcome Trust, the UK National Institute for Medical Research and Cancer Research UK. The Departments of Gastroenterology at UCLs hospitals of UCH and the Royal Free have very large patient bases with approximately 3,000 patients with Inflammatory Bowel Disease.

UCL has an excellent **Molecular Immunology Unit** with considerable experience of conducting trials of somatic gene therapy for various forms of PID including SCID-X1, CGD, ADA-SCID, and WAS. Other research interests include the pathophysiology of primary immunodeficiency syndromes especially WAS, the actin cytoskeleton in haematopoietic cells (with Dr Siobhan Burns), the development of somatic gene therapy (with Professor Bobby Gaspar, Professor Christine Kinnon, Dr Waseem Qasim, and for ocular disease with Professor Robin Ali), and thymus transplantation (with Dr Graham Davies). **Professor Adrian Thrasher** (https://iris.ucl.ac.uk/iris/browse/profile?upi=ATHRA78) would assist gene editing of bone marrow and subsequent transplantation were this to become feasible.

UCL has well-developed **Scientific Support Services**. These include DNA sequencing, microarray hybridisation and bioinformatic analysis, fluorescence-activated cell sorting (MoFlo high-throughput equipment), confocal and two-photon microscopy. These services are available to researchers across UCL at competitive prices.

UCL has an excellent **Biological Services Unit** (BSU/animal facility). Mouse husbandry is taken care of by BSU staff paid by UCL. Commercial testing for microbiological health status of the mice is carried out four times per year, paid for centrally by UCL.

Collaborators:

Professor Dario Alessi

Director of the MRC Protein Phosphorylation and Ubiquitylation Unit (PPU), Dundee. This is a major research centre that focuses on the understanding of the biological roles of phosphorylation and ubiquitylation and how disruption of these processes cause human diseases such as neurodegeneration, cancer, hypertension and immune disorders. The ultimate goal of the Unit's research programmes is to help develop new improved strategies to treat disease.
Dario's current main interest is in LRRK2 and its interaction with RAB proteins

Dr. Alexander Schmidt

Alexander's main interests are Mass spectrometry, Proteomics, Systems Biology. He is at the Biozentrum, which is the largest department at the University of Basel's Faculty of Science. The primary focus of this interdisciplinary institute is basic molecular and biomedical research and teaching. It has particular expertise in all aspects of mass spectrometry and proteomics. The Biozentrum holds a leading position nationally and internationally and closely networks with partners from the academic world and industry.

Professor Mathias Chamaillard

Centre d'Infection et d'Immunite de Lille. This is a centre of excellence in studies on infection and immunity, with particular reference to human disease. Mathias' particular interest is in determining the biochemical, anatomical and immunological features of commensals which overcome or preserve (either individually or as a whole organ) epithelial barrier function, and (ii) specific cellular and molecular features through which NOD2 and NLRP6 shape a protective assembly of commensal lineages against intestinal inflammation and tumorigenesis.

Professor Harry Hemmingway

Professor Hemmingway is director of the Farr Institute of Health Informatics Research is comprised of four nodes distributed across the UK and led from UCL, University of Manchester, Swansea University, and the University of Dundee. With a £17.5m-research award from a 10-funder consortium, plus additional £20m-capital funds from the Medical Research Council, the Farr Institute aims to deliver high-quality, cutting-edge research linking electronic health data with other forms of research and routinely collected data, as well as build capacity in health informatics research. The Farr Institute aims to provide the physical and electronic infrastructure to facilitate collaboration across the four nodes, support their safe use of patient and research data for medical research, and enable partnerships by providing a physical structure to co-locate NHS organizations, industry, and other UK academic centres.

Professor Dame Anne Johnson

Anne M. Johnson is Professor of Infectious Disease Epidemiology at University College London. Since 2014 she has been Chair of the UCL Population and Lifelong Health Domain and Vice-Dean for External and International Relations. From 2002-2010 she was Head of the Department of Primary care and Population Sciences and then Director of the Division of Population Health. She has considerable experience in population health issues.

Caroline Sabin

Professor of Medical Statistics and Epidemiology, Research Department of Infection and Population Health,
UCL. She initiated the UK Collaborative HIV Cohort (UK CHIC) Study, a major multicentre study of >30,000 HIV-infected individuals in the UK, one of the largest clinical cohorts of HIV-infected individuals worldwide. She is a member of the Scientific Oversight Workpackage for the EuroCoord collaboration – a major collaborative effort that involves all cohorts of HIV-infected persons in Europe (with information on >250,000 infected individuals).

Drs Nigel Field, UCH, Dan Ivens, The Royal Free Hospital and Alan Winston St Mary's hospital are all experts in the field of sexually transmitted infections and Professor Ailsa Hart, St Mark's hospital, is an expert on inflammatory bowel disease

Research proposal (B2 Ethical Issues Annex)

Please also specify any authorization or permission you already have for the proposed work and include copies. **UK Home Office Project Licence #70/8452** to Professor Anthony W Segal 2015-2020 “Microbicidal mechanisms of phagocytes and investigation of Inflammatory Bowel Disease”. Letter of authority attached.

IRAS form: The collection of blood, biopsies, saliva, intestinal fluid and surgical resection tissue from patients with inflammatorybowel disease and other inflammatory conditions to assess the functional effects of abnormal gene and protein expression

NHS R&D Form IRAS Version 3.0 Security of personal data Confidential Information Sheet

Several information and consent forms**Rationale for the use of animals**

The ultimate goal of the proposed experiments is to understand the molecular causes of Crohn’s disease and ulcerative colitis with the aim of developing diagnostic tests and effective therapy. Because the development of bowel inflammation is an integrated process requiring mucosal, vascular and immune responses, it is not possible to study the problem in dissociated cell culture or tissue slices and we must work with live animals.

Why mice?

Mice are the animal of choice because they are a well-developed genetic model, allowing us to manipulate cells in vivo using transgenic methodology and thereby gain insights about cellular and molecular mechanisms that would not otherwise be accessible. Their genome has been completely sequenced and there are extensive and resource banks freely-available, including cDNA clone libraries, bacterial artificial clone libraries, expression databases and the like. They are also amenable to experiments to test bowel inflammation and repair of disease predisposition by gene editing and transplantation. No other mammal offers this combination of advantages – in short it is one of the standard animal models for modern biomedical research. The mouse gastrointestinal tract has been shown to be a good model for understanding basic principles of bowel inflammation, including some human disease states.

The benefit and burden of the research on animals

Each mouse line must be maintained independently so the number of breeding colonies is relatively large and numbers of animals born also proportionally large. However, none of our mice should display a deleterious phenotype when maintained as pure lines. The expected phenotypes of mice carrying two mutations are also expected to be very mild and only expressed after the provocation of inflammation.

In view of these potential long-term benefits to humankind, the use of mice is, in my opinion, justified. It is also considered justified by the Home Office Veterinary Officer who scrutinises our License applications

Regulations safeguarding the welfare of animals in UK laboratories

The UK has a very highly regulated environment for scientific research using animals. All such work is regulated by the UK Government “Animals (Scientific Procedures) Act 1986”. This specifies in great detail:

(1) exactly what experimental procedures are regulated by law;

(2) the humane (“Schedule 1”) methods that must be employed to kill animals;

(3) which experimental procedures require a “Home Office License”;

Making and breeding genetically modified organisms is a regulated activity that requires a Home Office Project License that is held by the Project Leader (AWS in this case). This is a major document that must be renewed every five years. In addition, all individual researchers must hold a Personal License linked to the Project, that authorizes them to carry out specified procedures and confirms that the required training has been undertaken. A Home Office Project License that authorises all the procedures specified in this grant application is already in place. The current Project License expires in January 2020; a renewal application will be made in time to take over seamlessly from the existing License when the time comes.

Numbers of animals to be used and procedures for minimising the use of animals

We make rigorous efforts to use the minimum number of animals. This is achieved by different researchers sharing tissue from the same mouse wherever possible. We also try to minimise the proportion of newborn mice that are not useful by careful design of the mutant mouse breeding strategy. For example, we try to keep homozygous lines where possible because that reduces the discard of unwanted heterozygous pups, and also renders it unnecessary to take tail clips or ear punches for genotyping. Because of the nature of this project, relatively large numbers of mice are unavoidable. However, as pointed out above (section on “Benefit and burden”), most of the experiments are benign in that they mainly involve killing animals by a Schedule 1 method for harvesting of cells, or else exposing them to mild bowel inflammation.

Procedures to minimize animal suffering

All animals killed at the start of an experiment will be killed in the humane manner stipulated by the UK Government Home Office to minimize suffering. All mice are regularly inspected, at least once per day, and any that show any sign of distress for any reason are immediately killed by a humane method. UCL employs a Veterinary Inspector who will provide advice and assistance if there is a query or doubt over any matter relating to animal welfare.