The association between coeliac disease and factor V Leiden in the present family is strongly suggestive of the presence of a gene in linkage disequilibrium with the long arm of chromosome 1 which is able to trigger the development of CD in all carriers. We feel that further studies are needed to identify such a gene in order to unravel the pathogenesis of CD. Moreover, further assessment of the degree of association between CD and factor V Leiden may be clinically important to avoid severe episodes of thromboembolism as that which occurred in our patient.

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Urease induced calcium precipitation by *Helicobacter* species may initiate gallstone formation

Helicobacter species can colonise the mammalian gastrointestinal and hepatobiliary tract which usually results in a chronic infection coupled to an inflammatory host response. It is therefore not surprising that colonisation with *Helicobacter* species is linked with a range of inflammation associated gastrointestinal and hepatobiliary diseases.¹ Recently, this range has been expanded, with an association of infection with enterohepatic *Helicobacter* species and the formation of cholesterol gallstones.²

In their study, Maurer and colleagues² demonstrated that murine infection with the enterohepatic *Helicobacter* species *H. bilis* and *H. hepaticus* accelerated the formation of cholesterol gallstones in mice fed a lithogenic diet. Although the gallbladder mucosa in mice with gallstones displayed signs of inflammation, *Helicobacter* species were not cultured from the inflamed gallbladder or bile. Therefore, Maurer *et al.* hypothesised that the chronic immune stimulation caused by *Helicobacter* species, rather than a direct bacterial factor, led to the production of nucleating agents, thus indirectly linking *Helicobacter* species and cholesterol gallstone formation.² Although a role for inflammation cannot be excluded, we believe that identification of *Helicobacter* DNA in gallstones^{3,4} is

an indication that *Helicobacter* species may also play a direct role in gallstone formation.

Gallstones are crystalline bodies formed by accretion or concretion of bile components. Approximately 80% of gallstones are cholesterol gallstones, and 20% are pigment stones consisting of bilirubin and calcium, two components present in bile. There is a connection between the two types of gallstones as calcium-bilirubin salts form surfaces that are highly attractive for

cholesterols to adhere to.⁵ In addition, almost all types of gallstones contain a nidus of calcium.^{6,7} Bacteria have been shown to be able to initiate calcium precipitation and subsequent stone formation,^{8,9} similar to *Proteus mirabilis* and kidney stones.¹⁰ An important factor in bacteria induced stone formation is the enzyme urease which hydrolyses urea into ammonia and bicarbonate. Ammonia increases the pH and thereby favours generation of an insoluble form of

Figure 1 Urease activity is required for calcium precipitation by *Helicobacter* species. Top panel: Comparison of *H. hepaticus* strain ATCC51449, *H. pylori* strain NCTC12198 (left) and their isogenic urease negative mutants (right). Bottom panel: Comparison of wild-type *H. bilis*, *H. pullorum*, and *H. cinaedi* isolates. All *Helicobacter* species were grown on precipitation agar (Columbia agar supplemented with Dent selective supplement (Oxoid), 0.2% β -cyclodextrins, 333 mM urea, 100 μ M NiCl_2 , 20 mM NaHCO_3 , 187 mM NH_4Cl , 30 mM CaCl_2 , and 0.04% triphenyl tetrazolium chloride; all concentrations given are final concentrations). After overnight growth under microaerobic conditions (5% O_2 , 10% CO_2 , 85% N_2), calcium crystals were visible with urease positive *Helicobacter* species but not with urease negative *Helicobacter* species or with the isogenic urease negative mutants. Magnification 3 $\times$, photos taken with three megapixel digital camera. The figure represents a representative example of three independent experiments.

calcium and subsequent precipitation, and this may be a mechanism shared between different types of stones.

We noticed in the study of Maurer and colleagues² that cholesterol gallstone formation was only detected after infection with the urease positive species *H. hepaticus* and *H. bilis* but not with the urease negative species *H. rodentium* or *H. cinaedi*.² This prompted us to investigate whether *Helicobacter* urease activity is involved in precipitation of calcium. For this purpose we developed a precipitation agar that allows for simultaneous growth of *Helicobacter* species and testing of their ability to precipitate calcium. We tested four different calcium concentrations (30, 10, 5, and 1 mM CaCl₂); best results were seen at 30 mM of calcium (fig 1) but calcium precipitation also occurred at more physiologically relevant calcium concentrations (10, 5, and 1 mM)¹¹ although the crystals were smaller. All four urease positive *Helicobacter* species tested (*H. hepaticus*, *H. bilis*, *H. pylori*, and *H. mustelae*) were capable of precipitating calcium in our assay (fig 1) whereas isogenic urease negative mutants of three species as well as urease negative *Helicobacter* species (*H. pullorum* and *H. cinaedi*) were unable to do so (fig 1). Purified urease enzyme alone was also capable of precipitating calcium.

This suggests that urease positive *Helicobacter* species that are able to survive in or colonise the bile ducts (which excludes *H. pylori*¹²) may induce the formation of gallstones both directly via their urease activity and indirectly via the immune response. Our observations extend those previously reported,^{2-4, 12} and provide a possible mechanism to explain the association between hepatobiliary colonisation with urease positive *Helicobacter* species and gallstone formation.

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No association between the functional CARD4 insertion/deletion polymorphism and inflammatory bowel diseases in the German population

Inflammatory bowel diseases (IBD, OMIM 601458) are represented by two main subtypes, Crohn's disease (CD, OMIM 266600) and ulcerative colitis (UC, OMIM 191390). The first and most widely replicated susceptibility gene for CD is *CARD15* (caspase recruitment domain family, member 15) that encodes NOD2 (nucleotide binding oligomerisation domain protein 2), a protein involved in the pathogen associated molecular pattern recognition system (for review see Schreiber and colleagues¹). Identification of functional *CARD15* variants revealed the important role of impaired barrier integrity and host defence in the pathogenesis of CD and other inflammatory diseases.¹ Recently, an association between IBD and variants in the *CARD4* gene, which encodes NOD1, a structural homologue of NOD2, has been demonstrated in the British population.² In that study, McGovern *et al* identified the common (deletion) allele of the marker ND₁+32656 as a risk factor for IBD. Conversely, the minor ND₁+32656 allele, in haplotype combination

with its adjacent marker rs2907748, exhibited a protective effect.² ND₁+32656 may alter the splicing of *CARD4* by affecting the binding of an unknown nuclear factor.³ The more frequent deletion variant could result in a NOD1 protein with reduced numbers of the leucine rich repeats that are essential for pathogen recognition. The odds ratio (OR) associated with this allele was calculated to be 2.0.² Association of *CARD4* polymorphisms has also been observed with asthma and atopic eczema,^{3, 4} suggesting that *CARD4* may be a common barrier disease susceptibility gene.

In the present study, we evaluated whether the variants previously reported in the British population are also associated with IBD in Germany. In addition to ND₁+32656, we included the adjacent single nucleotide polymorphisms (SNPs) ND₁+27606 (rs2075822) and ND₁+45343 (rs2907748) in our analyses. ND₁+233 and ND₁+21984, which were only weakly associated with IBD in the study of McGovern *et al*, were not examined here. SNP genotyping was undertaken on a case control (1015 IBD patients (676 CD, 344 UC), 886 unrelated controls) and an independent family based panel (775 mother-father affected child trios for IBD (328 CD, 447 UC)) using TaqMan Assays-by-Design (Applied Biosystems, Foster City, California, USA). Given our sample sizes, our study provided >96% power to detect an OR of 2.0, assuming a risk allele frequency of 79% and 1% type I error. However, no significant association was detected with either the case control or independent family based CD/UC samples (tables 1, 2). McGovern *et al* also reported a strong association with specific disease subgroups.² We therefore stratified our sample for patients with early onset IBD (n = 449 <25 years) and patients with fistulae and stenoses (n = 491). Analyses of both subgroups showed no significant associations (data not shown).

In conclusion, there is no evidence for an association of the IBD phenotype with the previously reported *CARD4* susceptibility variants in the German population. The discrepant findings in the German and British samples may reflect true genetic heterogeneity, as has been demonstrated for *CARD15* associations.⁵ This heterogeneity is partly evident in the different linkage disequilibrium (LD) patterns of the three

Table 1 Summary of single marker association statistics for *CARD4* (caspase recruitment domain family, member 4)

Phenotype	Variant*	MAF _{controls} †	MAF _{cases} †	CCA‡	CCG§	TDT¶
				p Value	p Value	p Value
CD	rs2075822	0.81	0.80	0.569	0.308	1.000
	ND ₁ +32656	0.79	0.79	0.847	0.977	0.091
	rs2907748	0.79	0.77	0.420	0.578	0.306
UC	rs2075822	0.81	0.78	0.152	0.357	0.480
	ND ₁ +32656	0.79	0.76	0.124	0.295	0.951
	rs2907748	0.79	0.75	0.073	0.195	0.956
IBD	rs2075822	0.81	0.79	0.263	0.327	0.580
	ND ₁ +32656	0.79	0.78	0.510	0.804	0.248
	rs2907748	0.79	0.77	0.132	0.279	0.547

*All three assays had a call rate >95% and showed no significant deviations from the Hardy-Weinberg equilibrium in the control sample.

†MAF, major allele frequency in cases and controls.

‡CCA, case control analysis for alleles.

§CCG, case control analysis for genotypes.

¶TDT, transmission disequilibrium test.