Autophagy mediates ER stress and inflammation in Helicobacter pylori-related gastric cancer

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ABSTRACT

Autophagy is a cellular degradation mechanism, which is triggered by the bacterium Helicobacter pylori. A single nucleotide polymorphism (SNP) in the autophagy gene ATG16L1 (rs2241880, G-allele) has been shown to dysregulate autophagy and increase intestinal endoplasmic reticulum (ER) stress. Here, we investigate the role of this SNP in H. pylori-mediated gastric carcinogenesis and its molecular pathways. ATG16L1 rs2241880 was genotyped in subjects from different ethnic cohorts (Dutch and Australian) presenting with gastric (pre)malignant lesions of various severity. Expression of GRP78 (a marker for ER stress) was assessed in gastric tissues. The effect of ATG16L1 rs2241880 on H. pylori-mediated ER stress and pro-inflammatory cytokine induction was investigated in organoids and CRISPR/Cas9 modified cell lines. Development of gastric cancer was associated with the ATG16L1 rs2241880 G-allele. Intestinal metaplastic cells in gastric tissue of patients showed increased levels of ER-stress. In vitro models showed that H. pylori increases autophagy while reducing ER stress, which appeared partly mediated by the ATG16L1 rs2241880 genotype. H. pylori-induced IL-8 production was increased while TNF-α production was decreased, in cells homozygous for the G-allele. The ATG16L1 rs2241880 G-allele is associated with progression of gastric premalignant lesions and cancer. Modulation of H. pylori-induced ER stress pathways and pro-inflammatory mediators by ATG16L1 rs2441880 may underlie this increased risk.

Introduction

Helicobacter pylori is recognized as a class 1 carcinogen associated with gastric cancer.1 This pathogen contributes to the large majority of intestinal-type gastric adenocarcinomas (GC), which affects over 1 million patients each year.2 While the association between H. pylori infection and GC risk is irrefutable, the exact causative mechanism remains unclear. It is generally accepted that H. pylori-induced inflammatory processes resulting in morphological alterations of the gastric mucosa are a contributing factor, as GC is commonly preceded by premalignant lesions. The first lesion to be recognized is atrophic gastritis characterized by the disappearance of parietal cells and gland tissue. This can be followed by development of intestinal metaplasia (IM), defined by colonic features such as goblet cells and expression of intestinal markers.3,4 Finally, progression into malignant lesions such as dysplasia and GC can occur.

Despite the causative relationship between H. pylori virulence factors and gastric carcinogenesis, not everyone with H. pylori or gastric premalignant lesions will eventually develop GC. Therefore, interest in host factors influencing the gastric environment and gastric carcinogenesis has taken flight.5 One of these host factors potentially associated with development of GC is a single nucleotide polymorphism (SNP) in the gene ATG16L1.

ATG16L1 rs2241880 denotes a threonine to alanine change at position 300 (in which the A-allele corresponds to threonine or wild type and the G-allele corresponds to alanine or mutant), which causes a defect in autophagy,6,7 a cellular process in which un- or misfolded proteins from the endoplasmic reticulum (ER) are recycled. Importantly, autophagy can also be directed against intracellular pathogens and thus serves as an innate immune...
defense mechanism. Consequently, bacterial clearance is affected by ATG16L1 rs2241880. However, the role of autophagy in H. pylori infection appears to be complex as H. pylori may induce autophagy to promote its replication. Another key role for autophagy in the response to infection is the modulation of immune cell effectors, cytokine production and the control of inflammation. However, the exact ramifications of the autophagic abnormalities evoked by ATG16L1 rs2241880 on the innate immune response to H. pylori infection are unknown. This is further muddled by current conflicting findings from studies investigating the role of ATG16L1 rs2241880 and the risk of GC development in European and East Asian populations.

Autophagy itself can be either anti- or pro-carcinogenic depending on disease stage and metabolic requirements. Many tumor types show enhanced protein synthesis, which can cause ER stress by overloading of the ER with un-foldable proteins. Compensatory upregulation of the autophagic process to reduce ER stress is thus often observed in tumors. However, when autophagy fails to be properly activated, as is the case for ATG16L1 rs2241880, ER stress may be enhanced. Alternative ER stress alleviation comes from the unfolded protein response (UPR), aimed at temporarily blocking protein synthesis, and ER-stress markers and the UPR are indeed known to be induced in GC. The most important ER-stress biomarker, the unfolded protein chaperone GRP78, was associated with poor patient survival in a GC cohort comprising 328 patients.

Autophagy, ATG16L1 rs2241880, inflammation and ER stress have all been previously linked to H. pylori infection and GC, however, the interplay between these factors in gastric carcinogenesis is unclear. In this study, we aimed to investigate these processes in both gastric premalignant lesions and GC, in diverse ethnic groups.

**Table 1.** Association between ATG16L1 rs2241880 alleles and risk of gastric precancerous lesions and gastric cancer in two human populations.

| Population | Disease | Mutant Allele | Cases | Controls | OR | 95% CI | P |
|------------|---------|---------------|-------|----------|----|--------|---|
| Dutch      | IM      | A             | 234   | 4337     | 1.36| 1.12–1.65 | 0.0017 |
| Australian | GC      | A             | 206   | 5197     | 1.42| 1.04–1.95 | 0.0305 |

**Results**

**ATG16L1 rs2241880 G allele is associated with gastric cancer in an Australian population and this risk increases synergistically with Helicobacter pylori infection**

To substantiate the role of ATG16L1 rs2241880 in the GC cascade, this polymorphism was first investigated in Australian Caucasians presenting with GC (n = 117) compared to healthy controls (n = 232). The minor allele within this cohort is the G allele, therefore, analyses were conducted with the A allele as reference. Both G allele (OR: 1.42, 95% CI: 1.04–1.95) and mutant homozygosity (GG) (OR: 1.96, 95% CI: 1.03–3.69) were found to be risk factors for GC development in this population (Tables 1 and 2).

Since infection with H. pylori is central to GC etiology, we further evaluated the existence of a potential synergistic relationship between the polymorphism and infection on the risk of GC. This analysis showed that individuals who carried the G allele and were infected with H. pylori were the most at risk of GC as compared to those harboring the AA genotype, showing a substantial OR of 3.25 (95% CI: 1.25–8.09) (Table 3).

**ATG16L1 rs2241880 A allele is associated with mild gastric premalignant lesions in a Dutch population**

We then investigated whether ATG16L1 rs2241880 is associated with the GC precursor lesion IM in a Dutch IM cohort (n = 308) compared to the population-based cohort of the Rotterdam study I (n = 4542). The minor allele within this cohort is the A allele, therefore, analyses were conducted with the G allele as reference. A significant increased risk of developing premalignant lesions was found for individuals with an A allele (A allele – OR 1.36, 95% CI 1.12–1.65, AA genotype – OR 1.92, 95% CI 1.28–2.89, AG genotype – OR 1.71, 95% CI 1.20–2.47, dominant model – OR 1.77, 95% CI 1.26–2.49) (Tables 1 and 2). Because it is
Table 2. Association between ATG16L1 rs2241880 genotypes and risk of gastric precancerous lesions and gastric cancer in two human populations.

| Population | Disease | Mutant Genotype | Cases | Controls | Genotype frequencies | Genotype analysis |
|------------|---------|----------------|-------|----------|----------------------|-------------------|
|            |         | AA     | AG   | GG       | AA     | AG   | GG   | OR  | 95% CI      | P        | OR  | 95% CI      | P        | OR  | 95% CI      | P        | OR  | 95% CI      | P        |
| Dutch      | IM      | AA     | 56   | 122      | 42     | 975  | 2387 | 1405| 1.92 | 1.28–2.89  | 0.0017  | 1.71 | 1.20–2.47  | 0.0028  | 1.77 | 1.26–2.49  | 0.0008  | 1.33 | 0.97–1.81  | 0.0743  |
| Australian | GC      | GG     | 24   | 54       | 39     | 65   | 113  | 54  | 1.96 | 1.03–3.69  | 0.0427  | 1.29 | 0.73–2.33  | 0.3959  | 1.51 | 0.90–2.54  | 0.1526  | 1.65 | 1.02–2.71  | 0.0543  |
the ATG16L1 rs2241880 G allele that has been previously associated with GC, as also evidenced in our current Australian Caucasian cohort, we further subdivided patients into those whose gastric premalignant lesions were more severe (defined as an OLGIM stage III or IV) and those with mild premalignant lesions (OLGIM I or II). Interestingly, within this population we found that the A allele is only associated with an increased risk of mild premalignant lesions (A allele – OR 1.49, 95% CI 1.15–1.92, AA genotype – OR 2.48, 95% CI 1.38–4.52 and AG genotype – OR 2.32, 95% CI 1.38–3.84) as no significance was reached in either allele or genotype analyses of severe premalignant lesions (Tables 4 and 5).

### Functional consequences of ATG16L1 rs2241880 on gastric ER stress

Previous studies have shown that reduced autophagy in ATG16L1 rs2241880 G allele carrying cells may lead to increased levels of ER stress in intestinal epithelium. Therefore, we next investigated the functional consequence of this locus on GRP78 expression in antral biopsies of patients diagnosed with gastric premalignant lesions (from the Dutch cohort). Figure 1(a-b) shows that ER stress was indeed present in antral tissues of IM patients, and was mainly limited to the IM crypts (quantified in Figure 1(c)). Of note, the cell-type staining most positive for GRP78 are Paneth cells, which is in line with previous data showing that these cells are susceptible to ER stress (see example in Figure 1(d)). However, when patients were stratified according to their genotype, no association was observed between the ATG16L1 rs2241880 status and GRP78 expression in the antral biopsies (Figure 2(a-b)). Furthermore, we saw no significant differences in constitutive GRP78 expression between organoids (derived from IM patients from the Dutch cohort) carrying different variants of this ATG16L1 locus (Figure 2(c-d)). Thus, these data suggest that ER stress levels in gastric epithelium per se are not affected by ATG16L1 rs2241880 status.

### H. pylori reduces ER stress levels in gastric epithelium, while enhancing autophagy

As we observed enhanced ER stress levels in IM crypts, but no direct association with ATG16L1 rs2241880 status in either IM biopsies or organoids, we wondered whether ER stress would be modulated by *H. pylori* infection. Therefore, we examined the effect of *H. pylori* on the interrelationship between autophagy and ER stress in gastric cell lines. GES-1 and SK-GT-2 cell lines were stimulated with *H. pylori* in the absence or presence of tunicamycin, an antibiotic which

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**Table 3.** Joint effect analysis of *Helicobacter pylori* infection and ATG16L1 rs2241880 polymorphism on gastric cancer risk in an Australian Caucasian population.

| H. pylori status | Genotype | OR   | 95% CI   | P     |
|------------------|----------|------|----------|-------|
| (-)              | AA       | 1    |          |       |
| (-)              | G carrier| 1.11 | 0.38–3.07| > 0.9999 |
| (+)              | AA       | 2.12 | 0.69–5.73| 0.2058 |
| (+)              | G carrier| 3.25 | 1.25–8.09| 0.0210 |

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**Table 4.** Association between ATG16L1 rs2241880 alleles and the risk of mild or severe intestinal metaplasia (OLGIM) in a Dutch population.

| Disease subgroup | Mutant Allele | Allele frequencies | Allele analysis |
|------------------|---------------|--------------------|-----------------|
|                  |               | Cases  | Controls | OR  | 95% CI | P  |
| Mild IM (OLGIM I/II) | A             | G   | A       |     |        |     |
|                   |               | 107  | 133     | 5197| 4337   | 1.49| 1.15–1.92 | 0.0025 |
| Severe IM (OLGIM III/IV) | A             | G   | A       |     |        |     |
|                   |               | 99   | 101     | 5197| 4337   | 1.22| 0.93–1.61 | 0.1729 |

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**Table 5.** Association between ATG16L1 rs2241880 genotypes and the risk of mild or severe intestinal metaplasia (OLGIM) in a Dutch population.

| Disease         | Genotype frequencies | Genotype Analysis |
|-----------------|----------------------|-------------------|
|                 | Cases | Controls | AA vs GG | OR  | 95% CI | P  | AG vs GG | OR  | 95% CI | P  | Dominant model | OR  | 95% CI | P  | Recessive model | OR  | 95% CI | P  |
| Mild IM (OLGIM I/II) | GG   | AG   | AA       | 2.48| 1.38–4.52| 0.0002| AG   | 1.32| 0.84–2.04| 0.0003| 1.36| 0.88–2.04| 0.1693|
|                  | 71    | 31    | 1405 2387| 975 |      |       | 1.25| 0.76–2.04| 0.3998| 1.32| 0.84–2.09| 1.30| 0.81–2.615|
| Severe IM (OLGIM III/IV) | GG   | AG   | AA       | 1.50| 0.86–2.63| 0.1867| AG   | 1.25| 0.76–2.04| 0.3998| 1.32| 0.84–2.09| 1.30| 0.81–2.615|
induces accumulation of unfolded proteins.\(^{22}\) Stimulation with \(H.\) pylori for 16 hours did not affect GRP78 expression in GES-1 cells (Figure 3(a)) or SK-GT-2 cells (Figure 3(c)), however, in cells experiencing ER stress induced by tunicamycin, \(H.\) pylori but not \(E.\) coli, significantly reduced GRP78 levels. It is important to note that GRP78 levels were significantly increased in cells stimulated with \(E.\) coli when compared to cells stimulated with \(H.\) pylori, also suggesting that reduction of GRP78 is much less efficiently induced by other bacteria. We next investigated whether \(H.\) pylori also affects ER stress levels \textit{in vivo}. To this end, we compared GRP78 levels in patients with an active infection at time of biopsy sampling (\(n = 23\)) and biopsies of these same individuals after eradication treatment. ER stress levels were indeed significantly lower at time of infection with \(H.\) pylori (Figure 3(e-f)). Upon eradication of \(H.\) pylori, GRP78 increased to levels indistinguishable from patients who have never been infected with \(H.\) pylori (\(n = 24\)). These data suggest that GRP78 levels in IM crypts are negatively affected by the presence of \(H.\) pylori.

We hypothesized that \(H.\) pylori might induce autophagy and thereby resolve the ER stress induced by tunicamycin. Therefore, we investigated the activation of autophagy by determining the ratio of conjugated to unconjugated LC3B, an established autophagy marker. Interestingly \(H.\) pylori, but not \(E.\) coli, indeed appears to increase the LC3B II/I ratio in both unstimulated and tunicamycin-stimulated GES-1 (Figure 3(b)) and SK-GT-2 cells (Figure 3(d)). This effect appears to be specific to \(H.\) pylori as well as gastric epithelial cells, as no effect of \(H.\) pylori on ER stress was observed in either colon cancer or esophageal cell lines (Supplementary Figure S1).

**Figure 1. ER stress (GRP78) is increased in gastric IM tissues (taken by forceps biopsy from the gastric antrum).** Representative pictures of antral biopsies (\(n = 23\)) immunohistochemically stained for GRP78 (brown). (a) Normal epithelium of the gastric antrum, showing no IM. (b) Representation of a biopsy including mixed normal epithelium (left) and IM (right). (c) Quantitative analyses showing that IM tissues of the gastric antrum express more GRP78 than the normal epithelium as measured by Allred score. Statistical analysis was carried out using parametric t-test (two-tailed) with errors bars representing SEM (d) Magnification of individual crypt showing strong GRP78 staining located mainly in an intestinal Paneth cell, representing an IM patch within the biopsy. IM: intestinal metaplasia.
H. pylori-induced ER stress reduction may be correlated to ATG16L1 rs2241880 status

To assess the effect of ATG16L1 rs2241880 on H. pylori-reduced ER-stress, we studied gastric organoids individually derived from 10 IM patients with differing genotypes (4 GG, 3 AG, 4 AA). First, we verified that ER stress (basal levels of which are low in gastric organoids, see Figures 2(c-d) and 4a) could be induced by tunicamycin (Figure 4(a-b)). ER-stressed cells were subsequently treated with H. pylori. A non-significant increase in GRP78 expression ($p = .16$) was seen in organoids from G-allele homozygous individuals (Figure 4(c-d)). Conversely, a suggestive reduction of the LC3B II/I ratio in samples from patients with a GG genotype was seen ($p = .062$) (Figure 4(c-d)). These data demonstrate a trend toward reduced autophagy induction by H. pylori in individuals carrying the ATG16L1 rs2241880 G-allele.

ATG16L1 rs2241880 affects IL-8 production in gastric epithelial cells, but not in immune cells

We next sought to investigate other molecular consequences of carrying ATG16L1 rs2241880. For this, we used the ATG16L1 rs2241880 AG genotype-carrying AGS cell line to generate both ATG16L1 rs2241880 AA and GG daughter cell lines by
Figure 3. *Helicobacter pylori* reduces ER stress levels in IM tissues. (a-d) GRP78 (a, c) and LC3II expression (b, d) determined by Western blot analysis in GES-1 normal gastric epithelial cells (a, b) and SK-GT-2 gastric cancer cell line (c, d). Representative example as well as quantification of 4 individual experiments is shown. GRP78 expression and LC3B II/I ratio relative to B-actin and normalized against unstimulated sample, are shown. Statistical analysis was carried out using Mann-Whitney test (two-tailed) with errors bars representing SEM (e) Representative images of GRP78 staining of antral biopsies from a patient with active infection with *H. pylori* (E.I), the same patient 1 year after eradication of *H. pylori* (HP) (E.II), and an antral biopsy from a second patient who has not encountered *H. pylori* (E.III). (f) Bar graph of quantified Allred scores of the average of biopsies stained for GRP78 during infection (n = 24), after eradication (n = 23) and in patients who have never been infected (n = 23). IM: intestinal metaplasia.

CRISPR/Cas9 genome editing. We first assessed the autophagic flux in these gastric epithelial cells using p62 turnover assays. As expected, starvation induced autophagy while treatment with chloroquine inhibited this pathway (Supplementary Figure S2). Importantly, autophagy was impaired in GG-carrying cells when compared to AG- and AA-carrying cells, with increased accumulation of p62 with chloroquine and decreased degradation of p62 with starvation (Supplementary Figure S2), confirming our suggestive findings in gastric organoids generated from IM patients.

IL-8 production contributes to gastric carcinogenesis through inflammation-mediated tissue destruction, therefore, we investigated the effect of the three *ATG16L1* rs224180 genotypes on IL-8 production in these cells. Across all three genotypes we detected a significant upregulation in the expression of *IL8* following infection with *H. pylori*, the most dramatic of which was observed in GG-carrying cells, which demonstrated a 480-fold change (p = .0002) (Figure 5(a)). The AG- and AA-carrying cells in comparison elicited a modest upregulation of 6- and 18-fold, respectively. Importantly, at basal levels (i.e. no exposure to *H. pylori*), expression of *IL8* mRNA was 3-fold higher in the GG-carrying cells compared to the AA-carrying cells (p = .0310). The same phenomenon was also suggested when comparing with AG-carrying cells (p = .074). The above findings were mostly corroborated at the protein level (Figure 5(b)) and support the notion that disruptive autophagy promotes an increased IL-8 response in epithelial cells upon infection.24,25

We then turned our attention to the effect of *ATG16L1* genotype status on *H. pylori*-induced IL-8 production by PBMCs from healthy individuals with differing genotypes (4 AA, 6 AG and 6 GG). While IL-8 was clearly induced upon *H. pylori* stimulation, no difference in IL-8 production was observed between different genotypes (Figure 5(c)). This is in line with data showing no differences in
PBMC cytokine production in IBD patients with either the ATG16L1 GG or AA phenotype, and suggests that immune cells are not affected by rs2241880 status upon H. pylori stimulation.

**ATG16L1 rs2241880 modulates the production of other cytokines in gastric epithelial cells**

To further explore the extent of the role of ATG16L1 rs2241880 on the innate immune response to H. pylori infection in gastric epithelial cells, we investigated a profile of both pro- and anti-inflammatory cytokines including TNF-α, IL-1β, IL-10, IL-17A and IL-18.

Accumulating evidence suggests TNF-α is a key player in tumorigenesis, thus, we sought to evaluate its impact in H. pylori-driven gastric carcinogenesis and its effect under ATG16L1 rs2441880. At the mRNA level, we observed that H. pylori-infected AA-carrying cells exhibited a significantly higher fold change in TNFα expression compared to infected G-allele carrying cells (Figure 5(e)). Interestingly, upon infection, the G allele-carrying cells experienced a significant downregulation in TNFα expression, with the greatest fold change decrease demonstrated in homozygotes (0.48, p = .047). We legitimize these findings at the protein level (Figure 5(f)), where infected AA-carrying cells demonstrate the highest release of TNFα, while the infected AG- and GG-carrying cells show a significantly lower yield (both p = .011). These results are also in-line with previous studies which demonstrate that the GG genotype significantly impacts the production of TNFα in PBMC exposed to various bacteria and Candida.

The potent pro-inflammatory cytokine IL-18 is implicated in the pathogenesis of GC via its modulation of tumor cell survival and expression of metastasis-associated genes. Upon infection, we observed significant IL18 upregulation in our GG-carrying cells (Figure 5(d), p = .036), which is also significantly higher when compared across the other genotypes. We attempted to validate these findings at the protein level, however, production
Figure 5. ATG16L1 rs2241880 status does not affect IL-8 production in immune cells but modulates IL-8 and TNF-α production in AGS cells upon infection with Helicobacter pylori. (a) Expression of IL8 mRNA in H. pylori strain GC026-challenged edited and non-edited AGS cells. (b) Concentration of IL-8 in H. pylori strain GC026-challenged edited and non-edited AGS cells. (c) Isolated PBMCs derived from healthy Dutch volunteers (AA n = 4; AG n = 6; GG n = 4) release substantial amounts of IL-8 after 2 hours of stimulation with H. pylori, but this is not affected by donor ATG16L1 rs2241880 status. (d) Expression of IL18 mRNA in H. pylori strain GC026-challenged edited and non-edited AGS cells. (e) Expression of TNFA mRNA in H. pylori strain GC026-challenged edited and non-edited AGS cells. (f) Concentration of TNF-α in H. pylori strain GC026-challenged edited and non-edited AGS cells. Experiments with the AGS cells were conducted in triplicates. Statistical analysis was carried out using parametric t-test (two-tailed) with errors bars representing SEM. AA; wild-type homozygote, AG; heterozygote, GG; mutant homozygote.
of mature IL-18 in these AGS cells was extremely limited and statistical analysis determined no significant differences in expression of IL-18 across the different genotypes (Supplementary Figure S5).

A disadvantage of our employment of a gastric epithelial cell line was the limited expression of some of our immune targets. This was the case for IL-1β, IL-10 and IL-17A, which revealed non-amplifiable mRNA levels in AGS cells, as well as undetectable supernatant protein levels using a multiplex ELISA (not shown).

Discussion

Here, we investigated the role of the autophagy gene ATG16L1 in the progression of H. pylori-mediated gastric carcinogenesis. In carcinogenesis, autophagy may be exploited as a protective mechanism against apoptosis-inducing drugs or be downregulated in an effort to dysregulate cellular replication. In general, it is suggested that defective autophagy promotes tumorigenesis, with mutations in several autophagy-related genes occurring in GC. In addition to somatic mutations, germline genetic variants affecting the autophagy pathway may also contribute to gastric carcinogenesis. SNPs in genes regulating the autophagy lysosome pathway were significantly associated with GC in GWAS analyses. While genome-wide significance for any individual gene was not reached, the G-allele of ATG16L1 rs2241880, which renders autophagy less efficient, was associated with an increased risk for GC and H. pylori infection in a Chinese population. These findings are also consistent with a study demonstrating a positive correlation between mutant homozygosity (GG) and susceptibility to H. pylori infection in 162 Scottish and 483 German subjects. However, others were unable to find associations with ATG16L1 rs2241880 indicating that the exact role of host factors in H. pylori infection and subsequent gastric carcinogenesis remains elusive. In the current study, the G allele was associated with risk of GC within Australian Caucasians, and this risk was further exacerbated in H. pylori-infected individuals. GC is preceded by several premalignant lesions, which themselves are positively associated with H. pylori infection. Thus, we expected ATG16L1 rs2241880 to also be associated with these lesions. Interestingly, data from the Dutch population suggest that the G allele is not associated with development of early premalignant lesions. Overall, these results suggest that mild (OLGIM I–II) and severe (i.e. OLGIM stage III–IV, dysplasia and GC) disease stages are not similarly affected by ATG16L1 rs2241880. However, an important variable that was not addressed here was the presence and diversity of the infecting H. pylori strains. Virulence of the infecting H. pylori strains can influence disease outcome and severity, and within Scottish and German populations, ATG16L1 rs2241880 homozygosity conferred more susceptibility to vacA s1m1 strains compared to the s1m2 strains.

The ATG16L1 rs2241880 G-allele is thought to impair autophagy, which in itself is required for reducing cellular ER-stress. As such, ATG16L1 rs2241880 is associated with enhanced accumulation of unfolded proteins and ER stress markers in gastrointestinal disease. For example, in Crohn’s disease, where ATG16L1 rs2241880 represents a widely established genetic risk factor, the G-allele is associated with increased small intestinal ER-stress and GRP78 expression, most notably in intestinal Paneth cells. Interestingly, Paneth cells also appear during the development of complete intestinal metaplasia, and we found that ER-stress accumulation was most noticeable in these Paneth cells within IM tissues, although this was not associated with ATG16L1 rs2241880 status per se. We show that H. pylori reduces ER stress levels in vitro and ex vivo, possibly by induction of the autophagy pathway, and that this may be modulated by ATG16L1 rs2241880. This is in line with reports demonstrating that H. pylori activates and uses the autophagy pathway for its replication. However, this does raise the question as to why ER stress levels in general are upregulated in IM regions of the antrum. Even though IM may be caused by H. pylori-induced inflammation, the bacteria are unable to survive in the metaplastic crypts. Thus, ER stress induction in IM per se may be H. pylori-independent, which may partly explain why constitutive GRP78 levels in IM biopsies were ATG16L1 rs224180-independent. While metaplasia is not considered malignant, the
mutational burden is still increased when compared to the normal gastric epithelium, which in turn can increase ER stress by overloading the folding capacity of the protein synthesis apparatus, in particular in cell types with high-protein production such as secretory granule-rich Paneth cells.

We next sought to elucidate the molecular interactions between ATG16L1 rs2241880 and H. pylori-associated gastric carcinogenesis. Matching SNPs to their relevant cell type is imperative in order to understand their functional consequence. Previous studies on ATG16L1 silencing have demonstrated that the subsequent disruptive autophagy is associated with an elevated production of the important pro-inflammatory cytokine IL-8 in epithelial cells exposed to *Shigella* and *Haemophilus parasuis* SH0165.

We demonstrated that while *H. pylori* induced IL-8 production in PBMCs, this was not affected by ATG16L1 rs2241880 status. In contrast, IL-8 production was significantly enhanced in GC cells harboring the GG-genotype. These data suggest that rs2241880 has a predominant effect on the epithelial compartment, although we cannot exclude the possibility that the diversity in other immune-modulatory SNPs present in PBMCs from different donors, but not in gene-edited AGS cells, masked the effect of ATG16L1 rs2241880 on IL-8 production in these immune cells. Additionally, the effect may be cytokine-specific, as ATG16L1 rs2241880 affects *C. albicans*-stimulated TNFα, but not IL-8, IL1β, IL-6 or INF-γ production in PBMCs.

Nevertheless, an enhanced production of IL-8 by epithelial cells either constitutively or in response to *H. pylori* could contribute to an enhanced risk of carcinogenesis in GG-allele carriers, as IL-8 has been demonstrated to be an important factor in the sequence leading up to GC. It is of interest to note that IL-8-recruited granulocytes appear to play a role in IM and GC development, and that this may be independent of *H. pylori* infection.

This is in line with our results showing that the absence of infection still elicits a phenotypic difference in IL-8 production between the three ATG16L1 genotypes in AGS cells, which might independently contribute to carcinogenesis.

The role of the pro-inflammatory cytokine TNF-α in cancer is somewhat controversial with purported both anti- and pro-tumorigenic properties. Our data indicates that the ATG16L1 rs2241880 G allele reduces TNF-α levels in gastric epithelial cells when infected with *H. pylori*, while the A allele reports an increase. TNF-α production was also found to be decreased in PBMCs from patients harboring the GG genotype when stimulated with EIEC, *Listeria monocytogenes*, and *Salmonella typhimurium*, but not to other bacteria such as *Staphylococcus aureus* and *Mycobacterium avium paratuberculosis*, suggesting that not only does ATG16L1 rs2241880 modulate TNF-α but it functions in a pathogen-specific manner. Our data echoes previous results which highlighted that inhibition of autophagy in PBMC by 3 MA, an effect which may be mimicked by ATG16L1 rs2241880, also lead to a decrease in TNF-α production upon stimulation with LPS.

Interactions between TNF-α and autophagy are dynamic and two-way with both carrying the ability to regulate the other. Further studies should aim to elucidate the biological mechanisms underpinning the ATG16L1 rs2241880 impact on TNF-α production.

IL18 is constitutively expressed in epithelial cells and is synthesized as a precursor protein where the caspase-1 adaptor ASC (apoptosis-associated speck-like protein containing a caspase activation and recruitment domain) is required for maturation and cleavage of IL-18. While AGS cells can produce the precursor form of IL-18, they have been found to lack ASC and thus, are unable to produce mature IL-18. Our findings of limited IL-18 production in our AGS cells are, therefore, in concordance with the literature. We do show, however, that cells harboring the GG genotype have significantly increased levels of IL18 mRNA, the effects of which in other cell types may confer a tumorigenic advantage for GC metastasis and evasion of immunosurveillance in the host.

From these data, a model appears (Figure 6) in which a reduced autophagic capacity conveyed by the ATG16L1 rs2241880 GG-genotype contributes to gastric carcinogenesis through *H. pylori*-induced enhanced production of IL-8, decreased TNF-α levels, and enhanced ER-stress levels. In the absence of *H. pylori*, the carcinogenic process can be further driven in G-allele carriers by enhanced constitutive IL-8 levels. Overall, this work provides mechanistic insight into the role of genetic variants affecting autophagy in *H. pylori*-driven gastric carcinogenesis.
Methods

Population studies

Patient selection

Dutch patients presenting with gastric premalignant lesions were included from the ongoing prospective progression or regression of gastric premalignant lesions of the stomach (Proregal) cohort study\textsuperscript{59,60} (Supplementary Table S1). In total 308 patients were included for genetic analysis. For immunohistochemistry experiments, 47 patients were selected for whom a certified histological diagnosis of \textit{H. pylori} was available or who were negative for \textit{H. pylori} based on histology, serology and/or breath test. The study protocol was approved by the Institutional Review Board (MEC-2009-090), all patients signed an informed consent.

The Rotterdam Study is a population-based cohort of healthy individuals residing Rotterdam, details have been described previously.\textsuperscript{20} The cohort is representative of the Dutch population in a large city, comprising almost 6,500 healthy individuals aged between 45 and 75 years, with GWAS data available for \( n = 4542 \).

The Australian Caucasian study sample consisted of 117 non-cardia GC (NCGC) cases and 232 age-, gender- and ethnically-matched healthy controls (Supplementary Table S1). All subjects were of European ancestry (mainly from the United Kingdom, Greece, and Italy), aged between 40 and 70 years old, and resided in metropolitan Melbourne, Australia. Peripheral whole blood was collected between 1990 and 1994 as part of the ongoing Melbourne Collaborative Cohort Study (MCCS), where ethics approval was obtained by the Cancer Council Victoria (HREC 9500 and IEC 9001).

Human genomic DNA isolation and genotyping

For the Dutch IM population, DNA was isolated from whole blood using the Kleargene XL blood DNA extraction kit (LGC limited, Teddington, UK) and \textit{ATG16L1} rs2241880 was determined using Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) (see supplementary materials for details and Supplementary Figure S3 for examples). For the Australian population, genomic DNA was extracted from whole peripheral blood samples using the QIAamp...
Blood Mini Kit (Qiagen, Melbourne, Australia) and genotyped using matrix-assisted laser-desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) and the Agena Bioscience MassARRAY iPLEX assay (San Diego, USA) (see supplementary materials for details).

Statistical analysis
Deviation from Hardy-Weinberg equilibrium (HWE) for each population was tested using the chi-square ($X^2$) goodness of fit test. Analyses were carried out using the Fisher’s exact probability test (two-tailed $P$-values), and the statistical package GraphPad Prism version 8.0.2 (GraphPad Software Inc., San Diego, USA). A $P$-value less than 0.05 was considered statistically significant while values less than 0.1 indicated trend.

In-vitro studies
Cell lines
The GC epithelial cell lines SK-GT-2 and AGS, and immortalized gastric epithelial cell line GES-1 were cultured as described in supplementary material.

CRISPR/Cas9 Genome Editing
CRISPR/Cas9 genome editing technology was implemented to produce ATG16L1 rs2241880 knock-in AGS cell lines representing all three genotypes of the polymorphism (AA, AG, GG). For details, see Supplementary Material. Genome editing validation was performed using qPCR as well as Sanger sequencing and fragment analysis.

Organoids
Stomach organoids from antrum and corpus were cultured as previously published, see Supplementary Material and Supplementary Figure S4. For stimulation experiments, organoids were first mechanically disrupted after which the cell suspension was equally divided and stimulated with H. pylori. All experiments using organoids were conducted from passages 3–6.

Immunohistochemistry
Immunohistochemistry was performed as described previously and positivity for GRP78 was quantified using the Allredscore. See supplement for details.

Helicobacter pylori culture and infection assays
H. pylori strain 43504 (cagA+, vacA$^{s1m1}$) was acquired from ATCC (Wesel, Germany). The H. pylori strain GC026 (cagA+, cagE+, cagL+, cagT+, vacA$^{s1m1}$, babA+, oipA+, dupA+, and sabA+) was previously isolated from a Malaysian GC patient. As control, E. coli (strain DH5α) was used. Methods for infection assays are detailed in supplementary material.

RT-PCR analysis
Quantitative gene expression analysis (qRT-PCR) was conducted for the target genes TNFA, IL8, IL18, IL1B, IL10, and IL17A, normalized to the house-keeping genes glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and ribosomal protein lateral stalk subunit P0 (RPLP0). All primers were supplied by Sigma-Aldrich (Sydney, Australia) (primer sequences and PCR protocols can be found in the Supplementary Table S3 and Table S4). qRT-PCRs were performed independently for each gene using the SensiMix SYBR HI-ROX Kit (Bioline, Sydney, Australia) and the real-time PCR cycler Rotor Gene 6000 (Corbett Life Sciences, Sydney, Australia). The $\Delta\Delta CT$ method was implemented to quantify the relative gene expression of each cytokine in each subgroup.

Turnover assays and Western blot analysis
For turnover assays, ATG16L1 rs2241880 knock-in AGS cell lines were subjected to serum starvation (Earle’s Balanced Salt Solution (Sigma) supplemented with sodium bicarbonate 2.2 g/L) or chloroquine (40μM) (Sigma) treatment, for 6 hours.

Western blot was performed as described previously. After incubation with the primary antibodies (GRP78 #3177S, LC3B-I/II #2775S, p62 #5114S; Cell Signaling Technology), membranes were incubated with IRDye antibodies (LI-COR Biosciences, Lincoln, NE, USA) or Goat Anti-Rabbit IgG (H + L)-HRP Conjugate (BioRad). Detection was performed using the Odyssey reader or the ImageQuant LAS 500 (GE Life Sciences, Uppsala, Sweden). See supplement for details.

Enzyme linked Immunosorbent Assay (ELISA)
Supernatant was collected from cell cultures, aliquoted and stored at -20°C until analysis. IL-8 was assessed either by singleplex ELISA (ready-set-go
ELISA from eBioscience) or by a multiplex ELISA alongside targets IL-1β, IL-17A and IL-10 (Essential 4-Plex Human ProcartaPlex Panel 2 from ThermoFisher, Sydney, Australia). TNF-α and IL-18 were assessed using singleplex ELISAs (Abcam, Melbourne, Australia). All ELISAs were performed according to the manufacturer’s protocol.

Optical density (OD) measurement of plates was performed with either an infinite 200 pro ELISA reader (TECAN, Mannedorf, Switzerland) or the Dynamica HALO MPR-96 visible microplate reader (Dynamica, Gisbourne, VIC, Australia).

Statistical analysis
The data is represented by means ± SEM unless otherwise mentioned. Statistical analysis was performed using the student’s t-test, Mann Whitney U test or Fishers exact test (paired or unpaired, 95% confidence intervals, two tailed) using GraphPad (versions 5.0 or 8.0, GraphPad Inc, San Diego, CA, USA) and SPSS (Version 25, IBM, Armonk, United States).

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Data availability statement

Data supporting the findings of this study are available within the article and its supplementary materials.
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