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The C allele of the IL-1B-511 SNP is associated with higher risk for Gastric Cancer and its premalignant lesion: A prospective case-control study in Peru

D i s s e r t a t i o n

zur Erlangung des Doktorgrades

Dr. med.

(doctor medicinae)

an der Medizinischen Fakultät
der Otto-von-Guericke-Universität Magdeburg

vorgelegt von Sebastian Gehmert

geboren in Guben

Magdeburg 2008

GEWIDMET MEINEN ELTERN

UND MEINER FRAU

Teile dieser Dissertation wurden auf folgenden Kongressen präsentiert:

04/2008 125. Kongreß der Deutschen Gesellschaft für Chirurgie, Berlin
Sebastian Gehmert, F. Meyer, B. Velapatiño, P. Herrera, J. Cok, G. Vasquez, J. Combe, S. Wen, R. H. Gilman: Der „Single-Nucleotide"-Polymorphismus von IL-1B-511 erhöht das Risiko von atrophischer Gastritis und intestinalem Magenkarzinom bei *H. pylori* infizierten Patienten in Peru.

09/2006 3. Mitteldeutscher Chirurgenkongress (MDCV), Leipzig
Sebastian Gehmert, B. Velapatiño, P. Herrera, J. Balqui, L. Santivañez, J. Cok, G. Vasquez, J. Combe, D.E. Berg, D. Passaro, F. Meyer, R.H. Gilman: Einfluss des "Single-Nucleotide"-Polymorphismus von IL-1B- 511 & IL-1RN auf das Magenkarzinomrisiko bei *H.- pylori*- Infektion in Peru.

Desweiteren befindet sich ein Manuscript zur Publikation im Journal "American Journal of Tropical Medicine & Hygiene" im Reviewprozess (26.11.2008).

Sebastian Gehmert, B. Velapatiño, P. Herrera, J. Balqui, L. Santivañez, J. Cok, G. Vasquez, J. Combe, D. Passaro, S. Wen, F. Meyer, D. E. Berg, R. H. Gilman. The C allele of the IL-1B SNP is associated with elevated risk of premalignant lesion and gastric cancer in Peruvians with H. pylori infection.

Dokumentationsblatt

Bibliografische Beschreibung:

Gehmert, Sebastian:
The C allele of the IL-1B-511 SNP is associated with higher risk for gastric cancer and its premalignant lesion: A prospective case control-study in Peru.
-2008.- 74 Bl.: 12 Abb., 20 Tab.

Medizinische Fakultät der Otto-von-Guericke Universität Magdeburg
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Kurzreferat:
Das proinflammatorische Interleukin(IL)-1ß und sein korrespondierender Rezeptor IL-1RN spielen eine entscheidende Rolle bei der mukosalen Inflammation im Rahmen einer *H. pylori* Infektion. Das Ziel dieser Arbeit war es zu untersuchen, ob spezifische Polymorphismen mit einem erhöhten Magenkarzinom(Ca)risiko und seiner premalignen mukosalen Veränderungen (atrophische Gastritis) in Peru einhergehen. Dafür wurde eine hospital-based Fall-Kontroll Studie mit 658 prospektiven Patienten durchgeführt, wovon 419 für die statische Auswertung eingeschlossen werden konnten.
In der Studien-Population zeigte sich, dass Individuen die das C Allele des IL-1B-511 SNP tragen, ein erhöhtes Risiko sowohl für die chronisch-atrophische Gastritis (*P*=0,001) als auch für das Magen-Ca (*P*=0,002) besitzen. Im Vergleich dazu stand der IL-1RN- Polymorphismus in keinem Zusammenhang mit einem erhöhten Risiko, weder für das Magen-Ca noch der chronisch-atrophischen Gastritis.

Schlüsselwörter: [**IL-1B Polymorphismus**], [***H. pylori* Infektion**], [**Gastritis**], [**Magenkarzinom**], [**PERU**]

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1 ZUSAMMENFASSUNG

EINLEITUNG: Das proinflammatorische Interleukin(IL)-1ß und sein korrespondierender Rezeptor IL-1RN spielen eine entscheidende Rolle bei der mukosalen Inflammation im Rahmen einer *H. pylori* Infektion. Polymorphismen im IL-1RN Gen und in der Promotor-Region von IL-1B-511 haben eine veränderte IL-Produktion zur Folge und führen zu einer verminderten intragastralen Säureproduktion. Das ZIEL der Studie war es zu untersuchen, ob spezifische Polymorphismen mit einem erhöhten Magenkarzinom(Ca)risiko in Peru einhergehen, da Peru i) eine hohe Prävalenz der *H.- pylori*- Infektion, ii) eine hohe jährliche Inzidenz von Magen-Ca & iii) einen gemischten genetischen Pool in der Bevölkerung durch Immigration von Europäern, Asiaten, Nord-amerikanern & Amerindern aufweist.

MEDOTHIK: Es wurde eine hospital-based Fall-Kontroll Studie mit 658 prospektiven Patienten durchgeführt, wobei 419 Patienten in die statische Auswertung eingeschlossen werden konnten. Patienten mit nicht-atrophischer Gastritis (NAG, *n*= 158) dienten als Kontroll-Gruppe und wurden im Verhältnis 2:1 zu Patienten mit chronisch atrophischer Gastritis (ChAG, *n*=43) und im Verhältnis 1:1 zu Individuen mit Magenkarzinom (GC, *n*= 133) anhand von Alter, Geschlecht und ausgesetzten Umweltfaktoren zugeordnet. Eine *H. pylori* Infektion wurde durch histologische Untersuchung, PCR Technik und Kultivierung aus endoskopisch gewonnenen Biopsien nachgewiesen. Mittels PCR und Restriktion Fragmentanalyse (RFLP) wurden die Patienten erfolgreich für den IL-1B-511 SNP und der various number tandem repeat (VNTR) des IL-1RN genotypisiert.

ERGEBNISSE: Mit Hilfe konditional logistischer Regressionsanalyse zeigte sich in der untersuchten Population, dass der homozygote Genotype des IL-1B-511 C Alleles signifikant häufiger bei chronisch-atrophischer Gastritis (Odds Ratio [OR] = 11,2; 95 % Konfidenzintervall [CI] = 2.27-55.57) und Magen-Ca (OR = 4,15; 95 % CI = 1,33-12,93) auftrat als bei Patienten mit nicht-atrophischer Gastritis. Damit verbunden war ein knapp 6-fach erhöhtes Risiko für ein intestinales Magen-Ca im Corpus Bereich (OR = 6,3; 95 % CI 1,17-33,76) bei homozygoten C Allel Träger des IL-1B-511 SNP im Vergleich zu Kontroll-Patienten mit homozygoten T Allel Status. Im Vergleich dazu zeigte sich keine Assoziation zwischen dem IL-1RN-

Polymorphismus und einem erhöhten Risiko, weder für das Magen-Ca noch der chronisch-atrophischen Gastritis.

SCHLUSSFOLGERUNG: Das C Allel des IL-1B-511 SNP ist mit einem erhöhten Risiko sowohl für die chronisch-atrophische Gastritis als auch für das Magen-Ca in Peru verbunden. Wegen der hohen Prävalenz einer *H. pylori* Infektion kann der IL-1B-511 SNP ein Indikator für Patienten sein, die von einer umfangreichen Eradikationstherapie und intensiver, endoskopischer Überwachung von Mukosaveränderungen im Magen profitieren würden. In Studien mit Kaukasiern wurde das T- Allel mit einem erhöhten Magen-Ca-Risiko in Verbindung gebracht. Die vorliegenden Ergebnisse korrelieren mit Studien aus Asien, was auf eine populationsabhängige Assoziation hindeutet. Damit wird die Wichtigkeit vergleichender molekularbiologisch-epidemiologischer Studien deutlich, um Risikofaktoren in einer Population zu identifizieren.

Schlüsselwörter: [**IL-1B Polymorphismus**], [***H. pylori* Infektion**], [**Gastritis**], [**Magenkarzinom**], [**PERU**]

2 SUMMARY

BACKGROUND AND AIMS: It has recently been suggested that interaction between bacterial virulence factors and immune response of the host affect the outcome of *H. pylori* infection and may be influenced by polymorphisms in both, bacterium and host. Especially polymorphisms in IL-1B and IL-1RN genes were found to be associated with higher risk for gastric cancer development due to increased IL-1ß level in gastric mucosa in response to *H. pylori* infection. This thesis examines the relationship between proinflammatory interleukin gene polymorphisms, risk of chronic atrophic gastritis and gastric cancer in a developing country of South America with a high prevalence of *H. pylori*.

METHODS: A hospital-based case-control study among 658 prospectively recruited patients was conducted and 419 patients were included for further statistical analysis. Patients with non-atrophic gastritis (NAG, *n*=158), served as controls and

were matched 2:1 to patients with chronic atrophic gastritis, a premalignant lesion (ChAG, *n*=43) and 1:1 to subjects with gastric cancer (GC, *n*=133). Several biopsies were obtained endoscopically and *H. pylori* infection was determined by histological examination, PCR and cultivation. Patients were successfully genotyped for the IL-1B–511 SNP and the various number tandem repeats (VNTR) of the IL-1RN by using PCR and RFLP technique.

RESULTS: Using conditional logistic regression analysis adjusted for *H. pylori* infection, we demonstrated that homozygote C allele carriers of the IL-1B-511 SNP are associated with an increased risk for atrophic gastritis (odds ratio [OR], 11.2; 95 % confidence interval [CI], 2.27-55.37) and gastric cancer (OR, 4.15; 95 % CI, 1.33-12.93) as compared with the homozygote T allele variant. Stratification by location and subtype of gastric cancer in an unconditional logistic regression analysis revealed a higher risk for intestinal type of gastric cancer (IT-GC) in the corpus (OR, 6.3; 95 % CI, 1.2-33.8) for homozygous C allele carriers of the IL-1B-511 SNP. A higher risk was found for atrophic gastritis in the antrum region for the homozygous variant of the IL-1B-511 C allele (OR, 12.7; 95 % CI, 2.12-76.43). In comparison, the IL-1RN polymorphism was not associated with an increased risk for either gastric cancer or chronic atrophic gastritis in Peru.

CONCLUSION: This study is the first to verify the C allele of the IL-1B-511 SNP as a risk factor in a population from South-America. Similar to a population of China and unlike studies conducted with Caucasians, the C allele of the IL-1B-511 SNP appears to predispose individuals to ChAG and IT-GC. In view of a high *H. pylori* prevalence and a high recurrence rate of treated infection in Peruvians, IL-1B-511 SNP might be an indicator for patients who could benefit from rigorous anti *H. pylori* therapy and intensive monitoring of their gastric mucosal changes by intermittent gastroscopies. The findings suggest that the SNP in IL-1B-511 is a population-dependent risk factor and is involved in genetically predisposing for chronic atrophic gastritis and intestinal type of gastric cancer in Peru.

Key words: [**IL-1B polymorphisms**], [***H. pylori* infection**], [**Gastric cancer**], [**Gastritis**], [**PERU**]

3 ABBREVIATIONS

bp	Base pair
***cag*A**	Cytotoxicity associated gene A
ChAG	Chronic atrophic gastritis
CI	Confidence interval
DNA	Desoxyribonucleic acid
dNTP's	Desoxynucleictriphosphates
DT-GC	Diffuse type of gastric cancer
EDTA	Ethylendiamintetraacetate
EthBr	Ethidium bromide
Hp, *H. pylori*	*Helicobacter pylori*
HWE	Hardy-Weinberg Equilibrium
IL-1B	Interleukin-1-beta gene
IL-1ß	Interleukin-1-beta protein
IL-1RN	Interleukin-1 receptor antagonist gene
IL-1ra	Interleukin-1 receptor antagonist protein
INEN	Instituto Especializado de Enfermedades Neoplasicas
IT-GC	Intestinal-type gastric cancer
LOAYAZA	Hospital Arzobispo Loayaza
OR	Odds ratio
NAG	Non- atrophic gastritis
PCR	Polymerase chain reaction
PMNs	Polymorphonuclear neutrophils
RFLP	Restriction fragment length polymorphism
ROS	Reactive oxygen species
RUT	Rapid urease test
SNP(s)	Single Nucleotide Polymorphism(s)
UV	Ultraviolet
***vac*A**	Vacuolating cytotoxin-A
WHO	World Health Organization

4 INTRODUCTION

4.1 GASTRIC CANCER

While substantial efforts have been made to diagnose and treat gastric cancer, each year 870,000 new cases of stomach cancer are diagnosed worldwide, and it accounts for over 650,000 deaths annually [1]. The incidence differs greatly between Western countries and other parts of the world, and is highest in Japan, South America, Eastern Europe, and parts of the Middle East. Parkin *et al.* showed that 60 % of new gastric cancer cases occur in developing countries [2].

Worldwide, adenocarcinomas rank among the most frequent forms of gastric cancer (85 %) with approximately 40 % at the pylorus, 40 % within the corpus segment and 15 % at cardia. Due to asymptomatic or non-specific symptoms in its early stages, gastric cancer has usually already metastasized by the time symptoms occur, which is a reason for its poor prognosis. According to Lauren's classification, the histological distinction can be made between intestinal-type gastric cancer (IT-GC) and diffuse-type gastric cancer (DT-GC).

Depending on glandular architecture and cellular pleomorphisms, the intestinal-type gastric cancer is distinguished by 3 degrees of differentiation (good, moderate and poor). A multi-step process involving a progressive cascade of molecular and morphological changes has been proposed by Correa for the development of intestinal gastric cancer [3]. In this regard, intestinal metaplasia and atrophic gastritis are considered to be a background for intestinal-type carcinomas supported by epidemiological surveys [4-6].

In contrast, diffuse-type gastric cancer has no known pre-malignant precursor lesions as described for intestinal-type gastric cancer. Diffuse-type adenocarcinoma resembles signet-ring cell carcinoma as it is classified by WHO classification. Secreted mucus by tumor cells is delivered into the interstitium where large pools of mucus/colloid are produced which appear optically as empty spaces. Signet-ring cells emerge when the mucus remains inside the tumor cell. Furthermore, it is a poorly differentiated type of cancer and tumor cells metastasize by loss of adhesion to neighbouring cells.

4.2 PERU

Peru is one of the poorest countries of South America, where gastric cancer is the leading cause of death in both sexes [7]. Despite the fact that a decreased incidence of gastric cancer has been observed in developed countries, it increased to 24.3/100,000 in men and 17.6/100,000 in women in Peru between 1990 and 1997 [8].

Peru's population is composed of native Americans (Amerindians), Africans and Europeans, due to a migration from Siberia (13,000 years ago) as well as colonization from Europe and Africa in the last few hundred years [9]. Approximately 45 % of Peru's population consists of indigenous Amerindians and most are found in the Southern Andes. Since the beginning of the twentieth century, extensive internal migration has taken place and many Amerindians migrated from the highlands to the coastal region. Thus, a large portion of Amerindians can also be found in coastal cities, especially Lima. Mestizos are people of mixed European and indigenous Amerindian ancestry; around 37 % of Peruvians belong to this group. 3 % are from Africa and Asia and the remaining percentage of Peru's population is Criollo, a relatively unmixed European group which descended from colonizing Spaniards (Figure 1).

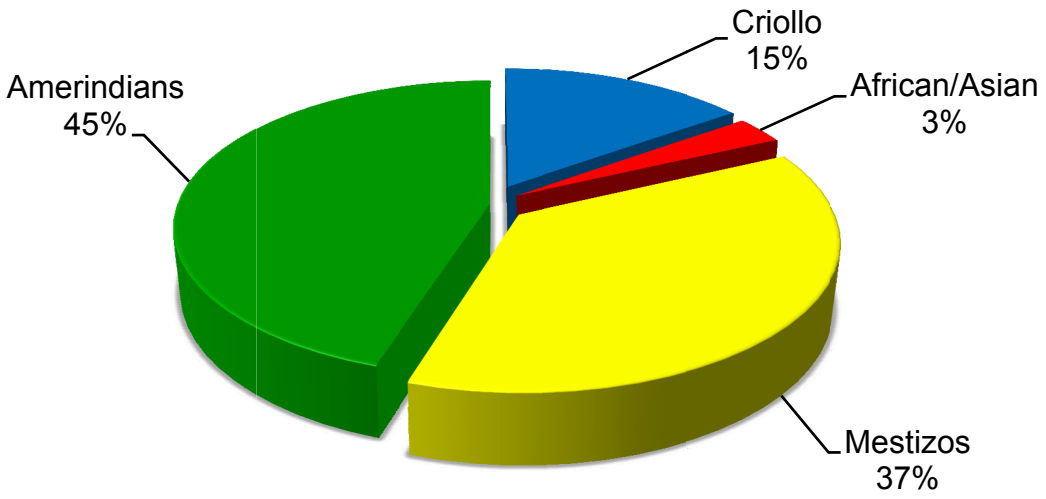

Figure 4.1: Population composition of Peru. Mestizos are people of mixed European and indigenous Amerindian ancestry. Criollos are a relatively unmixed European group which descended from colonizing Spaniards.

4.2.1 LIMA

Most residents of Lima, the capital town of Peru with almost 9 million inhabitants, belong to the group of Mestizos, a mixture of European and Amerindian background. Lima consists of more than 30 districts which differ greatly in infrastructure, public water supply, energy access, sewage system and waste disposal. Shantytowns, known locally as *asentamiento humano*, consist of informal and unguided construction of huts with absence of public sanitation and electricity (Figure 4.2.a).

(a) (b)

Figure 4.2: Informal, unguided house and hut construction in a shantytown district "*asentamiento humano*" of Lima (a). Rural areas are located in outlying districts of Lima and comparable with shantytowns with regards to living circumstances (b).

Rural areas, located in outlying districts of Lima, are comparable with shantytowns with regards to sanitary facilities and electricity (Figure 4.2.b). People of low socioeconomic status live in these areas and have no prospect of better living conditions due to limited funds. In contrast, residents with a higher annual income are able to live in districts where water is supplied by the Lima municipal system, waste is removed in a scheduled manner and house construction is comparable to standards of western countries.

Interestingly, the incidence of gastric cancer in Lima was found to be related directly with low socioeconomic level [8;10]. This suggests involvement of environmental factors in carcinogenesis. Even the WHO mentioned in the 2003 World Cancer Report that environmental factors are implicated in the cause of the majority of human cancers [11].

4.2.2 *H. PYLORI* INFECTION

Since the rediscovery of *H. pylori* by Warren in 1979, infection with this bacterium has been linked to several types of gastric cancer, including gastric adenocarcinoma and gastric lymphoma. Over 50 % of the world population harbours this bacterium but only a small proportion of infected individuals develop gastric cancer. Uemura *et al*. showed in a study with 1,246 endoscopically followed patients infected with *H. pylori* for an average of 7.8 years that only 3 % of them developed gastric cancer [12].

H. pylori is a genetically diverse bacterium with different strains subdivided by their expression of *cag*A and *vac*A. Individuals infected with cagA-positive *H. pylori* strains showed an increased risk for the development of gastric cancer [13]. However, in Peru around 85 % of the isolated *H. pylori* strains from previous sequencing studies were positive for proteins *vac*A and *cag*A, and no differences were found between patients with gastritis or gastric cancer [B. Velapatiño and R. H. Gilman, unpublished data]. Thus, it is quite plausible that other factors play an important role in Peruvians regarding the emergence and progression of precursor lesions (i.e. chronic atrophic gastritis, intestinal metaplasia, and dysplasia) that can lead to gastric cancer.

Nearly 90 % of adults in Peru undergoing upper-gastrointestinal endoscopy for dyspeptic symptoms are found to be infected with this bacterium [14;15]. In contrast, less than 25 % of young adults in North America and Western European countries carry *H. pylori* [16;17]. The high risk of this bacterial infection, relative to industrialized countries, is especially apparent in childhood. About one-half of Peruvian children become infected with *H. pylori* during their first years, and most others become infected within the following one to two decades [18;19].

Furthermore, *H. pylori* reinfection rate is very high in Peruvian adults after successful treatment with an antibiotic eradication regime [20;21]. Since infection of *H. pylori* starts in early ages and reinfection rate is high, this bacterium colonizes stomach mucosa for a long period of time in Peruvians.

Response to *H. pylori* infection comprised of an inflammatory and an immune component, characterized by cellular infiltration lead to chronic gastritis more or less pronounced. In addition, it has been shown that *H. pylori* infection increases mRNA level of IL-1B gene product and that gastric pH is more alkaline than in uninfected patients [22;23]. Previously, it was shown that gastric epithelial damage by *H. pylori* leads to a compensatory increase in mucosal proliferation in the antrum and corpus and that eradication of bacterial infection decreased the cell proliferation levels to normal after four weeks [24;25]. Cell proliferation returned to its previously high level in patients, in whom eradication had failed. Taken together, *H. pylori* and IL-1ß can lead to increased cell proliferation. An increased cell turnover itself might make the mucosa more vulnerable to mutagenic effects and toxic products in the inflamed stomach.

Along with the bacterium, cytokines and chemokines play a part in maintaining gastric mucosal inflammation and may have a significant impact in histopathological changes of gastric mucosa which apparently represent a continuum of changes from normal mucosa to carcinoma. Thus, inter-individual differences of cytokine production, which might be genetically determined, can play a decisive role in cancer susceptibility when *H. pylori* infection occurs.

4.3 POLYMORPHISMS IN THE IL-1 GENE CLUSTER

Genotype polymorphism is one of the molecular mechanisms that could cause an alteration in the expression and secretion of cytokines and could, therefore, play a crucial role in mediating different outcomes of *H. pylori* infection. Polymorphism can only affect a single nucleotide in a specific sequence and is therefore called as

single nucleotide polymorphism (SNP). It can occur by base substitution, insertion or deletion.

In addition, polymorphism can be related to a whole nucleotide sequence (10-100 nucleotides) that varies in the number of repetition (2-40 repetitions) and therefore entitled variable numbers of tandem repeat" (VNTR). Genetic variations are referred to as polymorphism once the frequency accounts to more than 1 % within a population.

SNPs make up to 90 % of all human genetic variations and can occur in coding and non-coding regions of genes, owing to the accidental manner of mutations. Due to the redundancy in the genetic code, SNPs within a coding sequence will not necessarily change the primary sequence of a protein or its function. Essentially, SNPs in non-coding regions can have consequences for gene splicing, binding of transcription factors or regulation of transcription with an alteration of the mRNA level. In this regard, a functional polymorphism denotes for instance an increased IL-1β production. This have been reported for a single nucleotide change at −511 positions [26] that is termed as IL-1B-511 SNP. The location of -511 represents a position 511 base pairs upstream of the promoter-starting site (Figure 4.3) and may manipulate a putative transcription factor binding site.

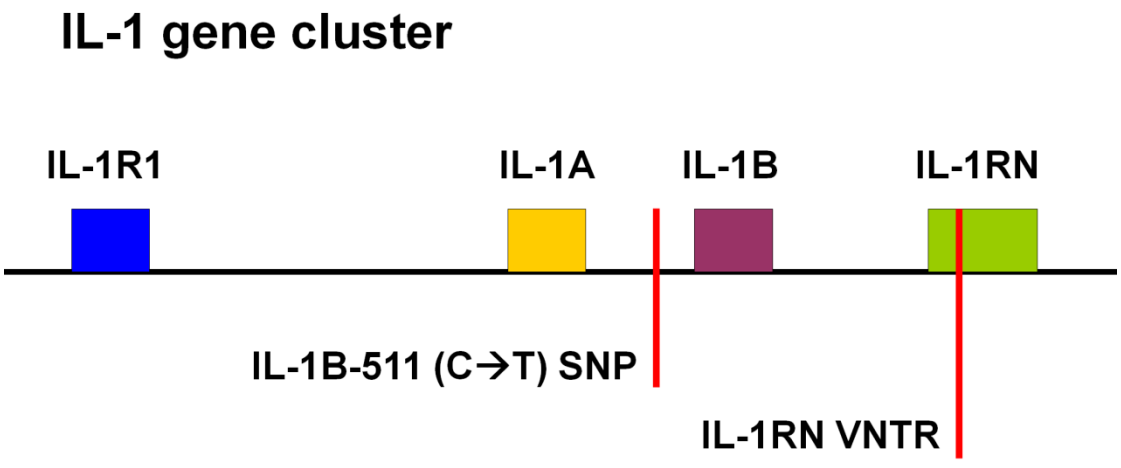

Figure 4.3: SNPs in the IL-1 gene cluster.
Overview of the IL-1 gene cluster with the SNPs in the IL-1B promoter region at position -511 and the variable number of tandem repeats (VNTR) in intron 2 of the IL-1RN gene.

IL-1ß plays an important role in the regulation of inflammatory responses, since it induces gene expression of other inflammatory cytokines. Beside the regulative role of IL-1ß in transcription, it also acts by inducing the proliferation of gastric epithelial cells in culture [27] and inhibiting the secretion of gastric acid [28]. A profound inhibition of the acid level raises the pH in the gastric mucosa and is responsible for progressive atrophy in the stomach [29]. The crucial role of IL-1ß to gastric acid output in stomach and subsequent progression to gastric cancer has been demonstrated with a transgenic mouse model. Overexpression of IL-1ß in gastric mucosa leads to lower gastric acid levels accompanied with severe gastritis followed by mucosal changes and adenocarcinoma [30;31]. However, the underlying mechanism of altered production of the proinflammatory IL-1ß cytokine by the IL-1B-511 SNP is not fully understood.

The effects of IL-1ß are mediated by its corresponding receptor (IL-1R) and subsequent activation of internal cell pathways. IL-1ra (gene, IL-1RN) shows the same affinity to the IL-1 receptor but does not induce any intracellular response. Thus, IL-1ra acts as a competitive inhibitor and subsequently modulates the proinflammatory effects of IL-1ß [32]. The IL-1RN gene contains a repetitive element of 86 bp (VNTR) in intron 2 (Figure 4.3 and 4.4), and at least 5 alleles of this polymorphism exist in human populations. A particular allele of this IL-1RN VNTR polymorphism (i.e. IL-1RN *2 allele) has been associated with a more severe clinical outcome in several diseases having an inflammatory component. The IL1RN *2 allele (2 repetitions) is associated with increased IL-1ß production in vitro [33]. Subjects with this proinflammatory genotype and *H. pylori* infection showed increased production of IL-1ß which leads to increased gastric inflammation, gastric atrophy and risk for developing gastric cancer [34].

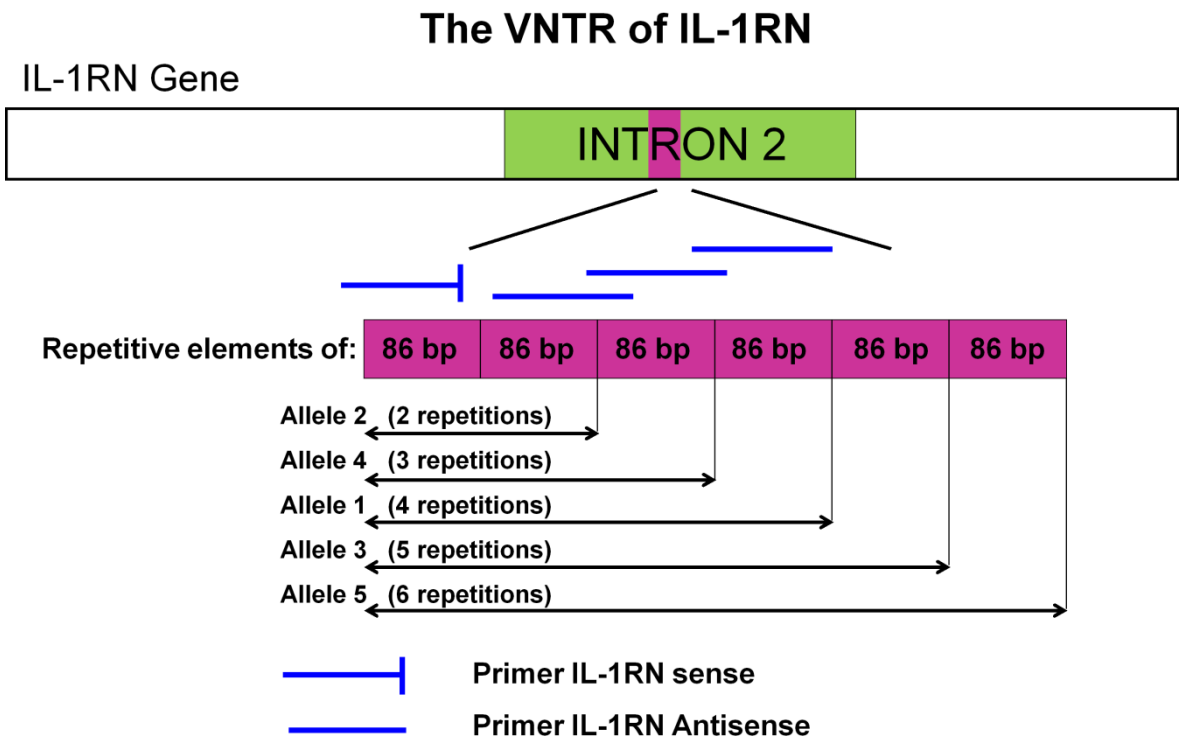

Figure 4.4: Overview of IL-1RN VNTR. The Intron 2 of the IL-1RN gene contains a penta-allelic 86-bp tandem repeat (VNTR, variable number of tandem repeats). Detection of genotype was carried out with PCR technique.

2.4 STUDIES

Since El-Omar *et al*. have reported a strong association between the IL-1B-511 SNP and the risk of gastric cancer [26;35], several studies were conducted to shed light on the impact of this single nucleotide exchange in different populations. Consistent associations between gastric disease and allele polymorphisms have been found for IL-1B-511*T (T allele) and/or IL-1RN*2 (*2 allele) carriers among Caucasians [26;35-43] and also among Asians [44-47]. However, not all of the subsequent studies were able to find an association between the distinct IL-1 gene polymorphisms and gastric cancer. In particular, further studies from Asia [29;48-57] failed to show an association compared to studies from Western countries [58;59].

Interestingly, a study from China reported unequal associations between IL-1B-511 alleles and gastric cancer risk even in the same population depending on the cancer prevalence [45]. Thus, a significant association between the IL-1B-

511 T allele and gastric cancer have been reported only for areas with a low prevalence of gastric cancer. Thus, the T allele frequency differed between the two healthy control groups and was highest for subjects from a high cancer prevalence area (51 %) compared to controls from a low cancer prevalence area (34 %). The same pattern could be observed when comparing reported T allele frequencies from different studies for healthy subjects (Figure 4.5a) and gastritis patients (Figure 4.6b). The T allele is more frequent (>46 %) in Asians and South Americans where the prevalence of gastric cancer is high. Furthermore, only few studies from these parts of the world reported a significant association between the IL-1B-511 T allele and gastric cancer.

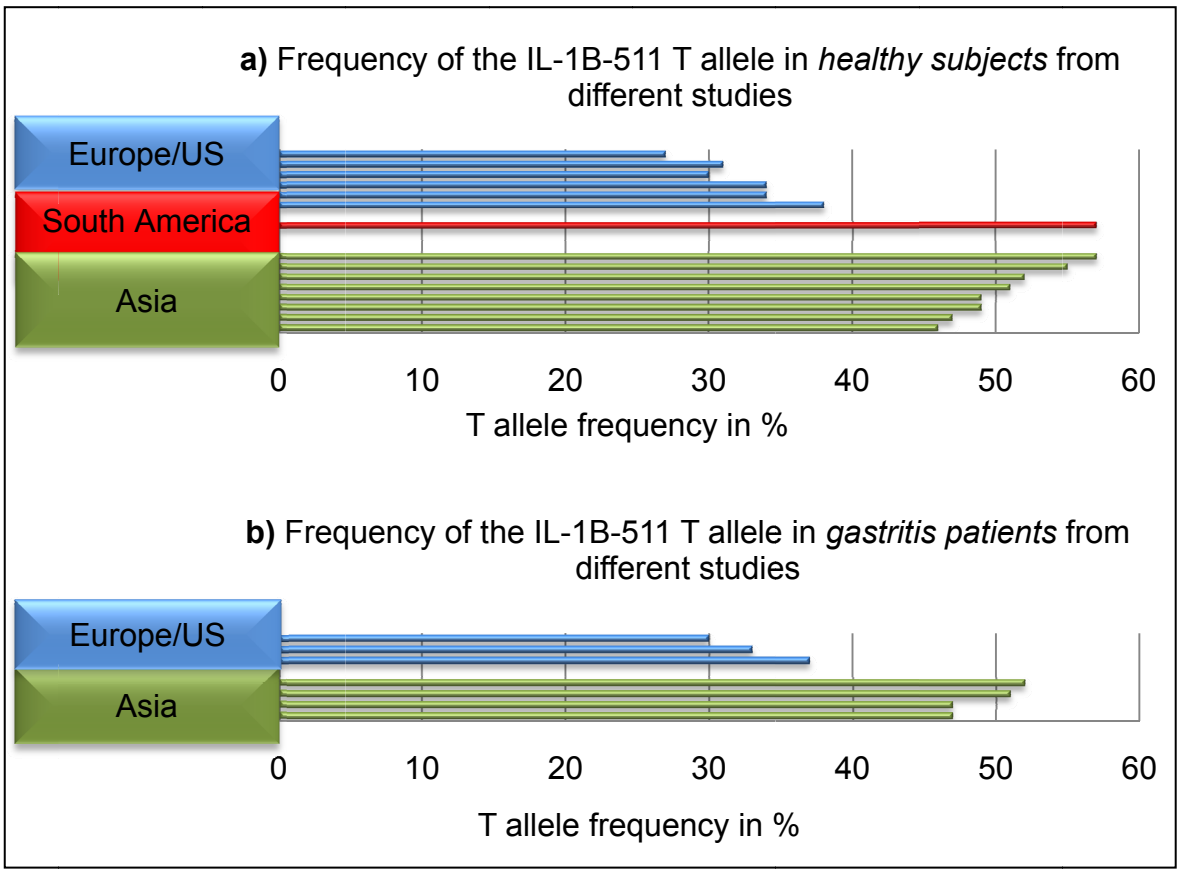

Figure 4.5: Frequency of the IL-1B-511 T allele in healthy subjects **(a)** and gastritis patients **(b)** from different studies. The T allele showed the highest frequency in Asia, where gastric cancer is very common and most reports fail to show a significant association between IL-1B-511 SNP and gastric cancer. **(a)** Studies from Europe/US [35;37;39;41;43;58] , South America [60], and Asia [29;50-52;54;55;57;61] **(b)** Studies from Europe/US [36;41;62] and Asia [46;48;51;63])

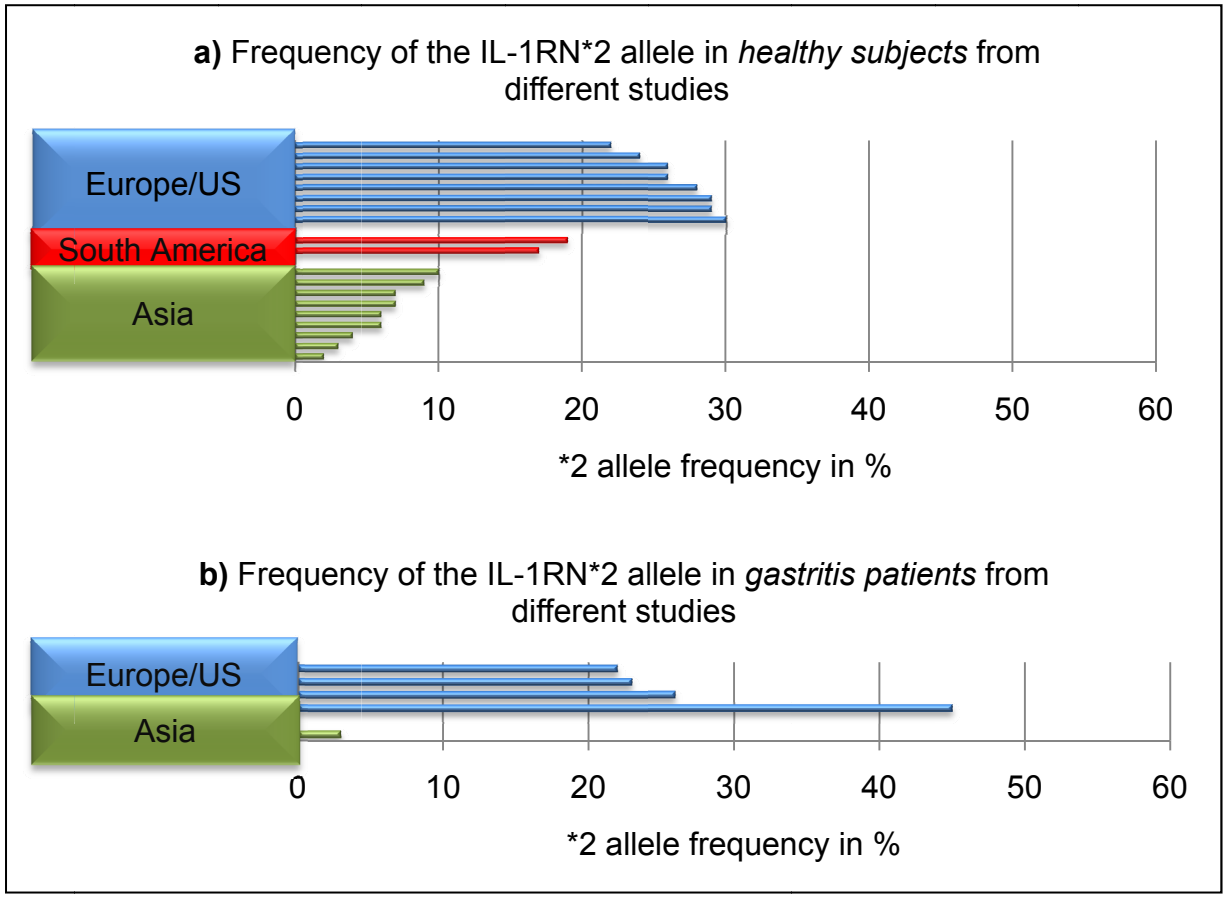

Figure 4.6: Frequency of the IL-1RN *2 allele in healthy subjects **(a)** and gastritis patients **(b)** from different studies. The *2 allele showed lowest frequency in Asia, where gastric cancer is very common and most reports fail to show a significant association between the IL-1RN VNTR and gastric cancer. **(a)** Studies from Europe/US [33;35;37;39-41;43;64], South America [13;60], and Asia [29;45;50;53-57;61] **(b)** Studies from Europe/US [36;41;59;62] and Asia [46])

Frequency of the IL-1RN *2 allele in healthy and gastritis patients showed comparable low rates of <10 % in studies from Asia (4.6 a and 4.6 b) where gastric cancer prevalence is high. In contrast, the reported *2 allele frequencies from Europe and US tend to have a frequency above 20 %.

Taken together, contradictory results from the literature regarding allele-association studies and varying cancer prevalence in different parts of the world underline the relevance of epidemiological polymorphism studies.

5 AIM OF THE STUDY

The aim of this study was to investigate whether or not alleles of the cytokine IL-1B gene (site -511) and of the IL-1 receptor antagonist (IL-1RN) are associated with an increased risk for chronic-atrophic gastritis or gastric cancer in the Amerindian population of Peru.

In particular, this thesis specifically aimed to find out whether:

1. There is a marked difference in the frequency of the investigated alleles in patients with gastric cancer compared with subjects having non-atrophic gastritis;

2. There is a difference between intestinal-type and diffuse-type gastric cancer with regards to the association to the distinct alleles in the IL-1 gene cluster;

3. The alleles show an association with the development of chronic atrophic gastritis, a known precursor lesion for intestinal-type gastric cancer;

4. Chronic-atrophic gastritis and gastric cancer arise predominantly at one site in the stomach in association with a distinct allele of the IL-1 gene cluster.

6 MATERIALS AND METHODS

6.1 MATERIALS

6.1.1 ASSAYS AND KITS

QIAamp DNA Mini kit	Qiagen, Valencia, CA, USA
Gram stain kit	BD Biosciences, San Jose, CA, USA

6.1.2 SUBSTANCES

Primer	Invitrogen, Carlsbad, CA,USA
Acetic acid	Sigma, St. Louis, MO, USA
Agarose gel (UltraPure)	Invitrogen, Carlsbad, CA,USA
*Ava*I restriction enzyme	NEB-Labs, Beverly, MA, USA
Bacto agar	BD & Co., Sparks, MD, USA
BHI agar and CNA agar	BD & Co., Sparks, MD, USA
BSA	Sigma, St. Louis, MO, USA
Cresol red	Sigma, St. Louis, MO, USA
DNA ladder (100 bp)	Invitrogen, Carlsbad, CA,USA
EDTA	Invitrogen, Carlsbad, CA,USA
Ethanol 96 %	Sigma, St. Louis, MO, USA
Ethidium bromide (1 mg/ml)	Fisher Scientific, NJ, USA
Gelatin	Centerchem, Norwalk, CT, USA
Glacial acetic acid	Sigma, St. Louis, MO, USA
Glycerol	Sigma, St. Louis, MO, USA
Haematoxylin and eosin solution	Sigma, St. Louis, MO, USA
Hydroquinone	Calbiochem, San Diego, CA, USA
Nucleotide mix	Invitrogen, Carlsbad, CA,USA

Proteinase K	Sigma, St. Louis, MO, USA
Silver nitrate	Res. Organics, Cleveland, OH, USA
Sodium acetate	Calbiochem, San Diego, CA, USA
Taq DNA polymerase (recombinant)	Invitrogen, Carlsbad, CA,USA
Tris base	Sigma, St. Louis, MO, USA
Urea agar base	BD & Co., Sparks, MD, USA

6.1.3 EQUIPMENT

BBL Campy Pouch microaerophilic system	BD & Co., Sparks, MD, USA
DC 120 camera with frame grabber	Kodak, Rochester, NY, USA
Dell workstation	Dell, Round Rock, TX, USA
Endoscope Fujinon EG450 HR	Olympus, Lima, Peru
Laminar flow hood, (NUAIRETM)	NUAIRE, Plymouth, MN. USA
Microcentrifuge VSMC-13	ProScientific, Oxford, CT, USA
Microwave (Sharp Carousel)	Sharp, Romeoville, IL, USA
Petri dishes (100 x 55 mm)	Fisher Scientific, Pittsburg, PA, USA
Seal-Rite 1.5 ml Eppendorf tube	USAScientific, Ocala, FL, USA
Tissue grinder	Bioworld, Dublin, OH, USA
UV illuminator	Kodak, Rochester, NY, USA
Vortex (Vortex Genie 2)	Bohemia, NY, USA
Water bath (Lab-Line 18020)	MIDSCI, St. Louis, MO, USA

6.1.4 SOFTWARE

Kodak digital science 1D software	Kodak, Rochester, NY, USA
SPLUS (Version 2000)	Insightful Corporation, Seattle, WA, USA
SPSS for Windows (Version 11.5)	SPSS Science, Chicago, IL, USA

6.2 METHODS

6.2.1 SETTING AND PATIENTS

A hospital-based case-control study was conducted between January 2005 and December 2006. All enrolled patients (over 18 years of age) had been i) newly hospitalized in two municipal hospitals of Lima/Peru (South America) and ii) undergone endoscopy of the upper gastrointestinal tract (inclusion criteria). Exclusion criteria were previous gastrectomy, concomitant major diseases, clinical cancer history, cardia cancer, atypical gastric cancer, MALT lymphoma, uncertain diagnosis or pregnancy.

Due to high rates of chronic *H. pylori* infection in Peru (in over 90 % of adults in lower socioeconomic classes) gastric biopsies without gastritis, typical of modern industrialized societies, are a rarity. Thus, patients with non-atrophic gastritis were used as the reference control group. Controls were frequency-matched to cases with respect to age (± 10 year), gender and environmental risk factors (housing material, water preparation, waste), showing significant differences between controls and cases. For cases with incident non-cardia gastric cancer (intestinal-type and diffuse-type), controls were matched 1:1; for cases with chronic atrophic gastritis, controls were matched 2:1.

All participants gave written consent for participation in this research study and were interviewed using a 130-item questionnaire that included personal characteristics, environmental risk factors, personal and family medical history. EDTA blood samples (4 ml) were obtained and stored at -20 °C for subsequent DNA extraction.

The study was in accordance with guidelines of the Declaration of Helsinki for Biomedical Research and was approved by the institutional ethics committees of The Johns Hopkins School of Public Health (Baltimore, MD, USA), and Asociación Benéfica PRISMA (Lima, Peru).

6.2.2 ENDOSCOPIC ASSESSMENT

All endoscopic examinations were routinely performed under mild sedation using an endoscope (Fujinon). For optimal assessment, specimens were taken as follows and shown in Figure 6.7:

- From the antrum within 2 to 3 cm orally from the pylorus: Greater (A1) and lesser (A2) curvature, respectively; *n*=2,
- From the corpus about 8 cm aborally from the cardia: Greater (C1) and lesser (C2) curvature, respectively; *n*=2,
- One from the incisura angularis (IA) along with all visible lesions, *n*=1+x

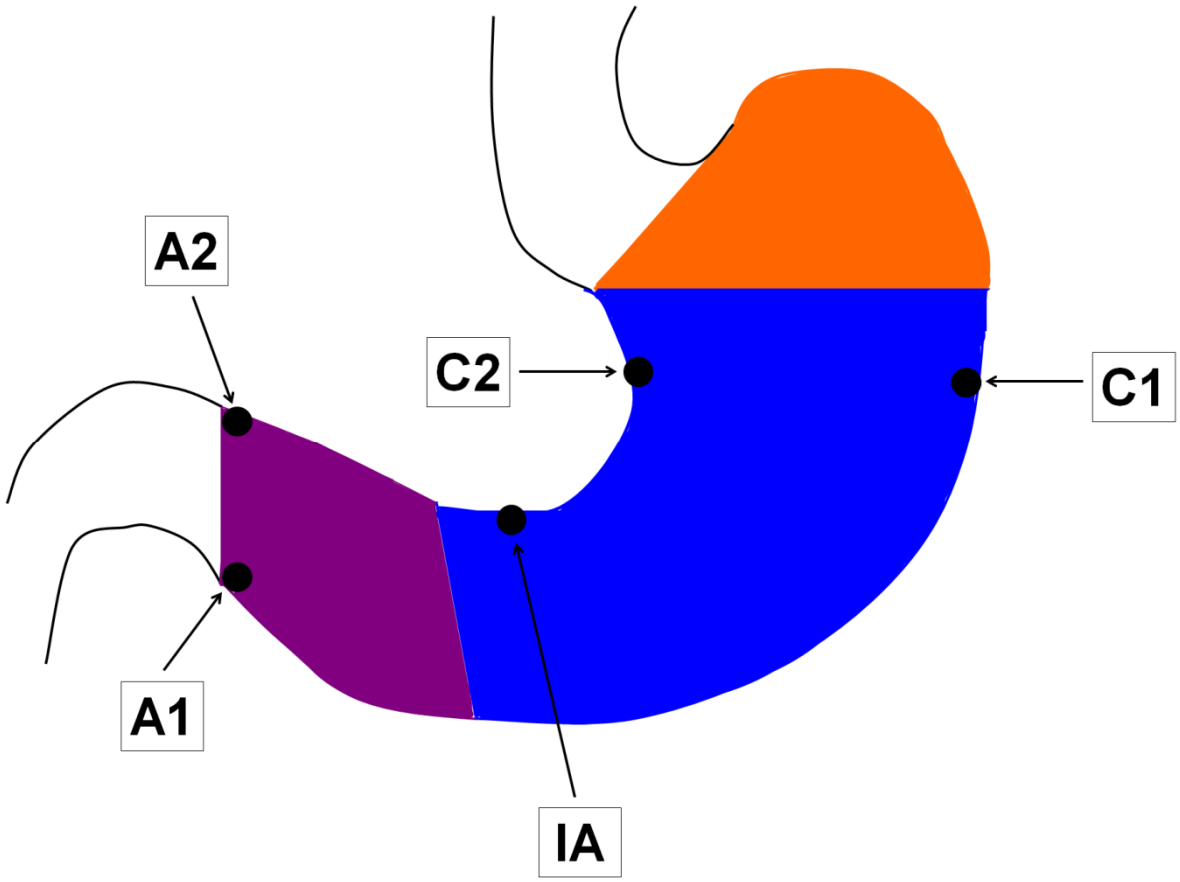

Figure 6.7: Schematic representation of the biopsies taken from each patient. One biopsy was taken from each site in the stomach, namely greater curvature of antrum (A1) and corpus (C1), lesser curvature of antrum (A2), corpus (C2), and incisura angularis (IA).

At least 3 biopsies along with all additional biopsies from visible lesions were subjected to histological preparation; one biopsy per patient was storage used for

culture and PCR essays. All samples were coded for anonymity and stored before processing for further investigation.

6.2.3 MOLECULAR BIOLOGICAL METHODS

6.2.3.1 DNA EXTRACTION

One biopsy from gastric antrum was mechanically ground using tissue grinder (Bioworld) in a 1.5 ml Eppendorf tube and lysed by adding 100 µl ATL and 20 µl proteinase K with subsequent incubation at 56 °C for 3 hours. The incubated mixture was occasionally vortexed to disperse the sample. 200 µl AL buffer was added and the sample was pulse-vortexed for 15 s and incubated at 70 °C for 10 minutes DNA was then precipitated by adding 200 µl of 96 % ethanol, pulse-vortexed for 15 s and applied to a spin column, as provided in the manufacturer's kit. During centrifugation at 6,000 g for 1 min, DNA was bound by a silica membrane of the spin column which separated genomic DNA from other extracts of the tissue. DNA bound by the silica membrane of the spin column was washed with 500 µl AW buffer and centrifuged at 6,000 g for 1 min. The wash step was repeated and the spin column was centrifuged at 10,000 g for 3 min. Finally, the DNA was eluted in a fresh 1.5 ml Eppendorf tube by centrifugation at 6,000 g for 1 min using pre-warmed 200 µl AE buffer. Extracted DNA was used to determine the genotype of IL-1B-511, IL-1RN and for *H. pylori* detection. For long-term storage, DNA was stored at -20 °C. When extracted DNA from biopsy yielded limited amount, stored blood samples from patients were used for DNA extraction.

6.2.3.2 POLYMERASE CHAIN REACTION

Over the past 20 years, PCR has become a standard technique to enzymatically amplify specific genome sequences. Three steps are required which are repeated several times and carried out using a PCR cycler machine. Starting at 95 °C, the double-stranded DNA denatures and primer can anneal in the second step, which must be optimized for each primer. For amplification, the Taq polymerase starts on the 3' end of each primer and adds dNTP's, reading the template from 5' to 3' at 72 °C. To perform several parallel reactions, a stock solution containing water,

buffer, dNTP's, primers and Taq-DNA polymerase was prepared in a single tube and then aliquoted into individual tubes. 2 µl of DNA from each patient was added and PCR, optimized for each primer (Table 6.1), were carried out on a PCR cycle.

Table 6.1. Primers for PCR

Primer (PCR product)	**Tm**[a]		**Sequence 5'- 3'**
IL-1B-511	50 °C	S[b]	TGG CAT TGA TCT GGT TCA TC
(305 bp)		A[c]	GTT TAG GAA TCT TTC CCA CTT
IL-1RN	55 °C	S	CTC AGC AAC ACT CCT AT
(3 products)*		A	TCC TGG TCT GCA GGT AA
UreB	67 °C	S	CGT CCG GCA ATA GCT GCC ATA GT
(436 bp)		A	GTA GGT CCT GCT ACT GAA GCC TTA

[a] Annealing temperature, [b] Sense primer, [c] Anti-sense primer
* 412 bp (four repeats: *1 allele), 240 bp (two repeats: *2 allele), 326 bp (three repeats: *3 allele)

6.2.3.3 GEL ELECTROPHORESIS

Agarose is a polysaccharide that is obtained from seaweed and when dissolved and heated in water, it melts as crosslinks are broken. Poured into a chamber and cooled, the crosslinks reform and the gel is set in the shape of the gel chamber. Thus, a matrix with defined pore size develops, dependent on the concentration of agarose, and can be exposed to an electric field. DNA fragments are separated by size due to DNA's negative charge in solution. Thus, large DNA molecules will migrate more slowly to the anode in the agarose gel in comparison to smaller molecules when loaded on the cathode.

Separated fragments can be visualized by adding ethidium bromide (EthBr) to agarose before gel casting. Due to the intercalating feature of EthBr, it binds strongly to DNA that migrates through the agarose gel in the process of electrophoresis. Under UV light, DNA-bound EthBr transmits energy which is visible as orange light. Using a 100 bp DNA molecular weight marker, the fragment size of the separated DNA can be determined.

The PCR product for IL-1RN and the RFLP for IL-1B were directly separated with 2 % UltraPure Agarose Gel. The following solutions were prepared (Table 6.2) and used for agarose gel preparation (Table 6.3):

Table 6.2. Preparation of 1 L Tris-acetate-EDTA (TAE) buffer (50X)

Reagents	Amount
Tris base	242.0 g
Acetic acid	57.1 ml
0.5 M EDTA	100.0 ml
ddH_2O	up to 1 L

Table 6.3. Preparation of an agarose gel

Reagents	Final concentration
TAE buffer (50X)	1X
Ethidium bromide	0.13 µg/ml
(w/v) Agarose	2 %

Subsequently, bands were visualized under an UV illuminator. A digital image was recorded with a charged couple device (CCD) camera and frame grabber using Kodak digital science 1D software on a Dell workstation. A 100 bp DNA ladder was used as a marker.

6.2.3.4 THE RFLP FOR GENOTYPING THE IL-1B-511 SNP

The restriction fragment length polymorphism (RFLP) technique utilizes the ability of restriction enzymes to cut double-stranded DNA containing a recognition sequence that is specific for each enzyme. The specific recognition sequence can be lost or arise spontaneously if a single nucleotide is changed at a specific site in the sequence. For this reason, RFLPs can be used for genotyping without sequencing the gene of interest.

For genotyping the IL-1B-511 SNP, a 305 bp fragment was initially amplified from 2 µl (10-20 ng) of the template DNA with PCR. 25 µl of the reaction mixture was prepared as follows (Table 6.5). Cresol red was used as loading dye (Table 6.4).

Table 6.4. Preparation of loading dye cresol red

Reagents	**Final concentration**
Glycerol	50 %
Cresol red	0.01 mg/ml

Table 6.5. Reaction mixture for PCR

Reagents	**Final concentration in 25 µl sample volume**
PCR buffer (10X)	1X
dNTP mix	0.25 mM
IL-1B-511 primer (sense)	0.5 µM
IL-1B-511 primer (antisense)	0.5 µM
$MgCL_2$	1.5 mM
BSA	0.1 µg/ml
Cresol red	0.1 mg/ml
Taq DNA polymerase	0.06 U

PCR was performed using the following cycling parameters (Table 6.6):

Table 6.6. PCR cycling parameters for IL-1B-511 primers

1x	Denaturation	95 °C	5 min
30x	Denaturation	95 °C	30 s
	Annealing	50 °C	30 s
	Elongation	72 °C	30 s
1x	Extenstion	72 °C	5 min

Afterwards, a RFLP was used to detect the absence or presence of the mutation at the -511 site of the IL-1B gene. Restriction analysis was conducted by incubating the prepared reaction mixture (Table 6.7) at 37 °C for 18 h.

Table 6.7. Reaction mixture for enzyme digestion

Reagents	**Volume**
PCR product	16 µl
*Ava*I enzyme (10,000U/ml)	1 µl
Enzyme buffer	2 µl
Water	up to 20 µl

Figure 6.8 shows a representative RFLP of the different genotypes compared with a 100-bp DNA ladder to identify the molecular size of the digestion product. Either an intact (*Ava*I un-cut) fragment of 305 bp (T allele) or two (*Ava*I cut) fragments of 190 bp and 115 bp (C allele) were obtained using electrophoresis. The following genotype notation was used: C/C (IL-1B-511 C homozygote), T/T (IL-1B-511 T homozygote) and C/T (IL-1B-511 heterozygote).

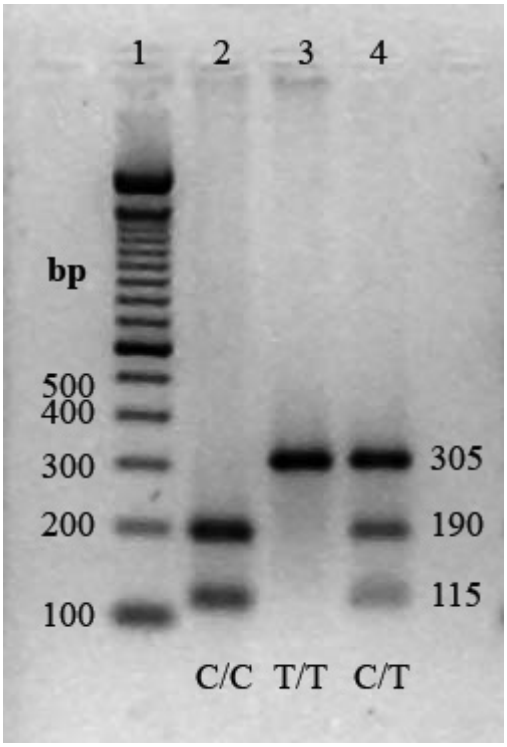

Figure 6.8: Detection of genotypes for the IL-1B-511 SNP. DNA amplicons were digested by the *Ava*I restriction enzyme, separated by gel electrophoresis and visualized under UV light. Three different patterns of IL-1B-511 genotypes were classified into C/C (homozygous C allele; lane 2), T/T (homozygous T allele; lane 3), C/T (heterozygote; lane 4). DNA ladder (100 bp) was used to determine the fragment size of separated DNA (lane 1).

6.2.3.5 GENOTYPING ASSAY FOR THE VNTR IN THE IL-1RN GENE

The diagnostic variable number of tandem repeat (VNTR) marker for IL-1RN alleles were genotyped by PCR with similar sample preparation and cycling parameters as used for IL-1B-511 genotyping but optimized with 3 mM $MgCl_2$, and annealing temperatures (T_m) of 55°C for IL-1RN primers. The IL-1RN alleles were coded as follows: *1 allele (four repeats of the 86-bp region yields a 412-bp PCR product), *2 allele (two repeats, 240 bp) and *3 allele (three repeats, 326 bp).

For statistical analysis, this polymorphism was treated as bi-allelic using a short allele category with two repeats (*2 allele) and a long allele category (L allele) with three or more repeats (*1 allele and *3 allele). Figure 6.9 shows the pattern of the genotypes 1/1, 2/2, 1/2 and 3/2 compared with a 100-bp DNA ladder.

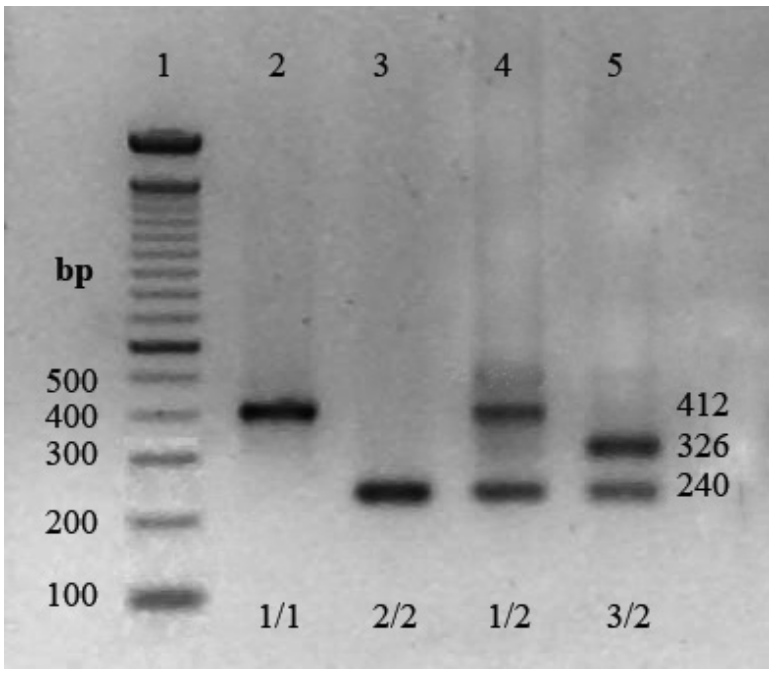

Figure 6.9: Agarose gel after electrophoresis shows different genotypes for the VNTR in the IL-1RN gene. Separated DNA fragment sizes were classified into 1 (four repeats, 412 bp), 2 (two repeats, 240 bp), 3 (three repeats, 326 bp). For statistical analysis three or four repeats were categorized as L allele (homozygous L allele; lane 2) and 2 repeats as *2 allele (homozygous *2 allele; lane 2, heterozygote; lane 4+5). DNA ladder (100 bp) was used to determine the fragment size of separated DNA (lane 1).

6.2.3.6 INTERNAL ASSAY CONTROL

The efficiency of each genotyping assay has a bearing on the determination of the genotype itself. Thus, the absence of the restriction recognition site for *Ava*I can also be due to the mal-function of the restriction enzyme. For this reason it is crucial to ensure that each run of an assay reveals the same results and is working properly.

To validate the performance of each assay setting for the IL-1B-511 SNP and IL-1RN VNTR, one fifth of samples from the preceding setting were reanalyzed in the subsequent run. Furthermore, several samples were sequenced for the polymorphism and for every assay all possible genotypes (C/C, C/T, T/T for the IL-1B-511 SNP and *2/*2, *2/L, L/L for the IL-1RN VNTR) served as internal positive controls. All results were completely concordant.

6.2.4 PATHOHISTOLOGY

One biopsy each from the antrum, corpus and incisura were fixed in 10 % neutrally buffered formalin, embedded in paraffin, subsequently cut in 3-µm thick sections and followed by haematoxylin and eosin staining for routine pathohistological investigation according to standard protocol. Each slide was interpreted by an experienced but blinded senior pathologist without knowledge of the area of origin of the microsection, clinical data or endoscopic characteristics. Grades of gastritis were classified according to the updated Sydney Classification System [65]. These included superficial gastritis, deep chronic gastritis, and chronic atrophic gastritis. Diagnoses of superficial gastritis and deep gastritis were merged and termed as non-atrophic gastritis (NAG).

Gastric carcinoma patients were subdivided according to Laurén's classification as carcinomas of the intestinal, diffuse or mixed (atypical) type. Each subject was diagnosed based upon the most severe or pronounced finding revealed in the pathohistological investigation of the gastric biopsy.

6.2.5 DIAGNOSIS OF *H. PYLORI* INFECTION

H. pylori infection was detected by culture, silver stain and PCR for *ure*B of gastric biopsies. Biopsies were considered positive if any of these three tests was positive and negative when all three tests were negative.

6.2.5.1 PCR SPECIFIC FOR *H. PYLORI* UREASE GENE

Extracted DNA from the antrum tissue sample was used to detect *H. pylori* with PCR using primer specific for a 463-bp segment of the *ure*B (urease) gene (Table 6.1). The same mix of reagents was used as for genotype assay (Table 6.5) with the following exceptions (Table 6.8):

Table 6.8. Regents deviating from genotype assay reaction mixture for PCR

Reagents	**Final concentration in 25 µl sample volume**
*Ure*B primer (sense)	0.4 µM
*Ure*B primer (antisense)	0.4 µM
$MgCl_2$	2.5 mM
Taq DNA polymerase	0.02 U

PCR was performed using the following cycling parameters (Table 6.9):

Table 6.9. PCR cycling parameters for *Ure*B primer

1x	Denaturation	95 °C	5 min
35x	Denaturation	95 °C	60 s
	Annealing	67 °C	60 s
	Elongation	72 °C	60 s
1x	Extenstion	72 °C	5 min

6.2.5.2 CULTURING OF *H. PYLORI*

One biopsy from the gastric corpus was homogenized and suspension was used for *H. pylori* culture on petri plates containing brain heart infusion (BHI) agar and CNA agar. Plates were incubated inverted under microaerophilic conditions (O_2, 5 %; CO_2, 10 %; N_2, 85 %) at 37ºC and 100 % humidity for 4-7 days by use of BBL Campy Pouch Microaerophilic system. When visible colonies appeared (Figure 6.10), they were identified for *H. pylori* by positive urease test and Gram`s stain.

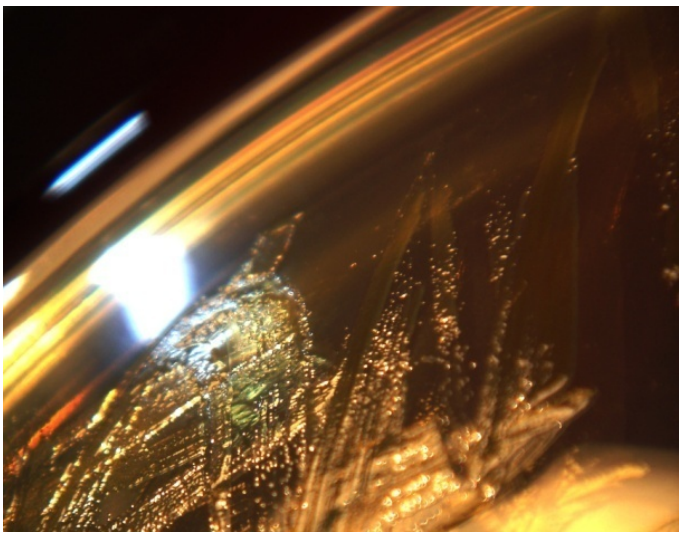

Figure 6.10: *H. pylori* cultured on agar plate.

RAPID UREASE TEST (RUT)

Urease produced by *H. pylori* catalyzes the hydrolysis of urea to ammonia. This activity of urease can be detected with urea agar base. The resulting ammonia raises the pH of the medium which contains phenol red causing a color change from yellow to red. The agar base was prepared as follows:

Urea agar base (1.5 g) and Bacto Agar (1.5 g) were suspended in 95 ml distilled water and dissolved by boiling for 5 min. Agar mix was sterilized by autoclaving, and 5 ml sterile 40 % urea solution was added when cooled to 50 °C. Urea agar base enriched with 40 % urea was aliquoted in sterile 1.5 ml Eppendorf tubes and was allowed to set in the slope position and was refrigerated at 2-8 °C prior to use.

Colonies that had to be identified from agar plate were placed on urea agar base and considered positive when color changed after 20 minutes.

GRAM`S STAIN

Bacterial cell wall is composed of peptidoglycans that can be penetrated by crystal violet, a cationic dye, forming blue-black complexes with iodine in the protoplast when flooded with iodine solution. Gram-negative bacteria have a thin peptidoglycan wall and an additional layer consisting of lipopolysaccarides that is

external to the peptidoglycan wall. Acetone makes the lipopolysaccharide wall more permeable and crystal violet can be washed out, allowing subsequent uptake of the basic fuchsin stain that gives Gram-negative bacteria (*H. pylori*) a red color. Gram-positive remain blue-black due to the trapped iodine dye complexes in the bacterial wall.

To test colonies, a smear sample was heat fixed on a glass slide and stained as follows:

Steps	**Time**
ddH_2O	briefly
Crystal violet	10 s
ddH_2O	briefly
Lugol‘s iodine	10 s
ddH_20	briefly
Acetone	1-2 s
Water	briefly
0.25 % Basic fuchsin	15 s

6.2.5.2 WARTHIN-STARRY SILVER STAINING

Warthin-Starry silver staining was additionally performed to Hematoxylin and Eosin (HE) staining, using biopsies obtained from the antrum, corpus and incisura angularis. 3 µm paraffin sections were deparaffinized, hydrated with deionized water and then rinsed in Walpole’s buffer. Slides were covered with 1 % silver nitrate and microwaved (600 watts) at a medium setting for 1.5 min and incubated in this solution for 5 min. Afterwards, slides were rinsed in Walpole’s buffer (Table 6.10) and then flooded with freshly prepared developer solution (Table 6.11). Warm water was used to rinse slides before dehydrating in two changes of 95 % ethanol followed by two changes of absolute ethanol. Finally, slides were cleared in xylene and mounted with permount. Each biopsy was scored negative or positive for *H. pylori* and classified for the density of bacteria as low (1, <20 per view field), moderate (2, <99) or extensive (3, more >99).

Walpole's buffer was used to solve silver nitrate, gelatine, and hydroquinone.

Table 6.10. Walpole's buffer

Reagents	Amount
5 M acetic acid	17.6 ml
5 M sodium acetate	2.4 ml
ddH_20	filled up to 500 ml

Table 6.11. Developer

Reagents	Amount
2 % silver nitrate	1.5 ml
3 % hydroquinone	0.5 ml
Gelatine	7.5 ml

6.2.6 DATA COLLECTION AND STATISTICAL ANALYSIS

Data obtained from the questionnaire were entered initially into a Microsoft EXCEL database using double data entry, to ensure accurate records. Databases were merged and checked for congruence and exported to SPSS for statistical analysis. Figure 6.11 shows an overview of procedures done for every case and control who participated in this study. Descriptive statistical analysis was calculated, including proportions, percentage, means, and standard deviations. A paired, two-sided *t*-test was used to compare differences in continuous variables for paired data. To calculate odds ratios (ORs) and 95 % confidence intervals (CIs) for IL-1B-511 and IL-1RN genotypes, a conditional logistic regression model was applied, adjusted for *H. pylori* infection. Stratification in an unconditional logistic regression analysis was used to evaluate whether the association between the cytokine polymorphisms and gastric cancer risk differed by histology type of gastric cancer or location of diagnosis. The dependent variable in this study was the histological diagnosis.

Odds ratios (OR) were estimated under a co-dominant model (homozygote and heterozygote genotypes that carry the variant allele versus homozygote wild type, the designated reference group). All statistical analyses were performed with SPSS software and SPLUS. Differences were considered statistically significant if $P<0.05$. All reported confidence intervals (CIs) are 95 % and P values were exclusively two-sided.

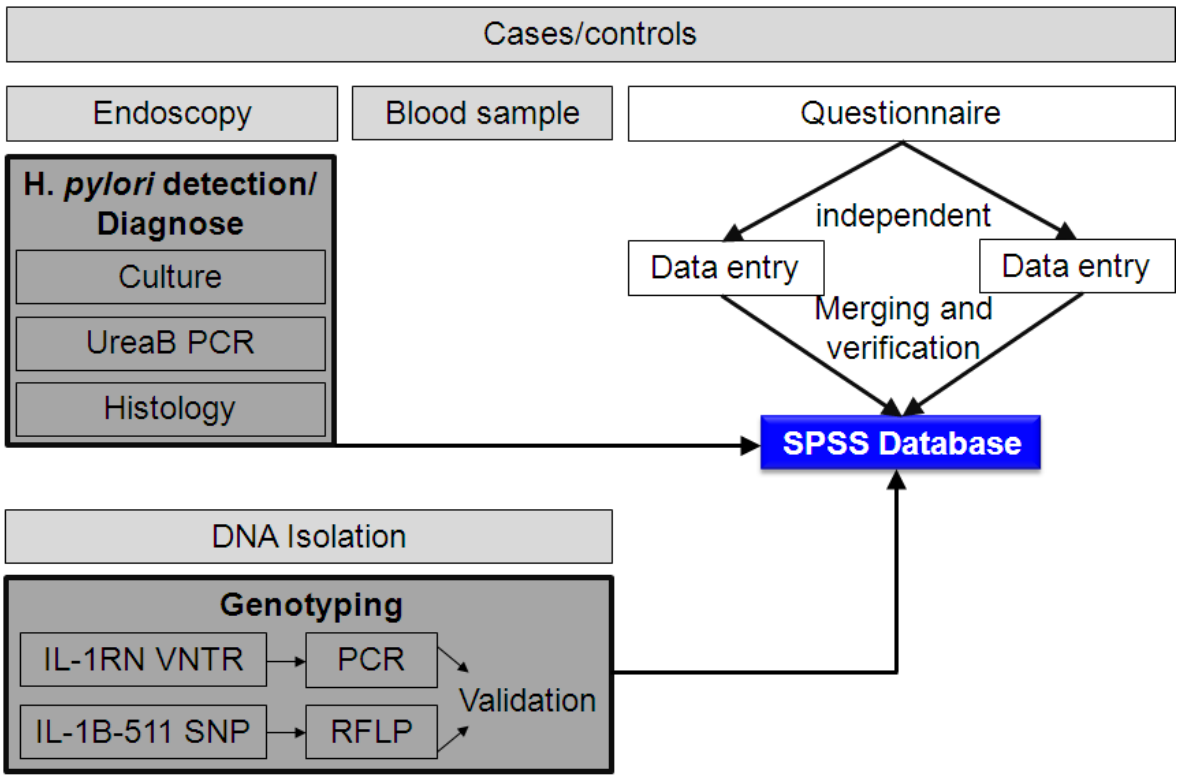

Figure 6.11: Overview of methods and procedures in the study.

HARDY WEINBERG EQUILIBRIUM (HWE)

The Hardy-Weinberg law suggests no changes for allele and genotype frequencies in a population and its successive generations. A deviation from the HWE may indicate, for instance, a selection process or gene flow. Hardy-Weinberg equilibrium of alleles at individual loci was assessed using an internet based program available at http://ihg2.helmholtz-muenchen.de/cgi-bin/hw/hwa1.pl.

7 RESULTS

7.1 STUDY POPULATION

Overall, 658 consecutive patients with gastritis or gastric cancer were enrolled in this study. Of those, 419 patients diagnosed for non-atrophic gastritis (NAG), chronic atrophic gastritis (ChAG) and gastric cancer (GC, intestinal and diffuse type) were used for further analyses (Figure 7.12).

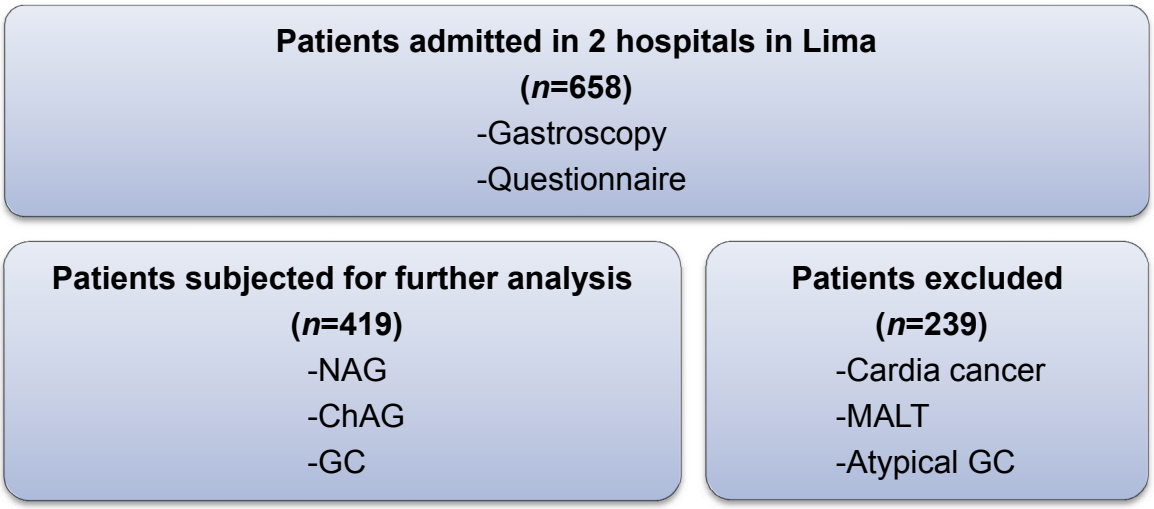

Figure 7.12: Overview of study population and number of patients that were subjected to further analysis, including diagnoses of non-atrophic gastritis (NAG), chronic atrophic gastritis (ChAG) and gastric cancer (GC, but only diffuse and intestinal type).

Patients were recruited in two municipal hospitals (i.e. Hospital Arzobispo Loayaza; LOAYAZA and Instituto Especializado de Enfermedades Neoplasicas; INEN) of Lima and no significant difference regarding age, sex, diagnoses and *H. pylori* infection were revealed (Table 7.12). Therefore, patients from both hospitals were merged for statistical analysis. All patients were Mestizos having a genetic background from Amerindians and Europeans.

Table 7.12. Comparison between the two municipal hospitals of Lima regarding age, sex, *H. pylori* infection and diagnoses. All *P* values were above the significant level of 0.05 indicating no significant differences for patients from both hospitals.

Characteristics	HOSPITALS LOAYOZA	HOSPITALS INEN	*P* value
Total number	263	156	
Mean age years (SD)	62.59 (14.86)	60.34 (14.83)	0.14†
Male/female ratio	0.88:1	0.77:1	0.544*
H. pylori infection	81.7	80.1	0.70*
Diagnoses, n(%)			
NAG	126 (47.9)	65 (41.7)	0.27‡
ChAG	34 (12.9)	15 (9.6)	
IT-GC	63 (24.0)	48 (30.8)	
DT-GC	40 (15.2)	28 (17.9)	

SD Standard Deviation
† T-test
* Fisher´s Exact Test
‡ Pearson Chi Square

7.2 CASES AND CONTROLS

Superficial or deep gastritis were combined to non-atrophic gastritis (NAG) and diagnosed for 191 patients that served as control group. Gastric cancer (only intestinal and diffuse-type) was histologically confirmed in 179 patients that had a mean age not significantly different to the control group (gastric cancer, 61.85 years; controls, 60.34 years). More women were diagnosed for cancer as well as for non-atrophic gastritis and sex ratio revealed no significant difference between these two diagnoses (*P*=0.3) (Table 7.13).

49 patients were diagnosed for chronic atrophic gastritis (ChAG) and their mean age of 66.9 years significantly differed (*P*=0.004) from that of controls. In addition, more males were found in the case group of ChAG than in controls but the difference did not reach a significant level (*P*=0.054). The proportion of *H. pylori* positive patients was highest in the non-atrophic gastritis group (88 %), reflecting the high prevalence of this bacterial infection in Peru. The infection rate of *H. pylori* was lower in patients diagnosed with chronic atrophic gastritis (79.6 %)

and even diminished significantly (*P*=0.001) in the gastric cancer group (74.2 %) when compared to non-atrophic gastritis patients.

Table 7.13. Characteristics of all cases and controls (*n*=419) that were subjected to further analysis.

Characteristics	Controls (NAG)	Cases (ChAG)	*P*	Cases (GC)	*P*
Total number	191	49		179	
Mean age years (SD)	60.34 (14.36)	66.90 (13.45)	**0.004**†	61.85 (15.52)	0.33†
Male/female ratio	0.71:1	1.33:1	0.054*	0.88:1	0.30*
H. pylori infection	88 %	79.6 %	0.16*	74.2 %	**0.001***

Non-atrophic gastritis (NAG), chronic atrophic gastritis (ChAG), gastric gancer (GC)
SD Standard Deviation
* Fisher´s Exact Test
†T-test

Much of the epidemiological data from the questionnaire showed a significant difference between cases and controls as revealed by unconditional logistic regression adjusted for age, sex and *H. pylori* infection (Table 7.14). The percentage of gastric cancer patients having no refrigerator (35.7 %) was more than two-fold higher than in the control group (15.2 %). A higher percentage was also found for chronic atrophic gastritis patients that stored food without cooling (22.2 %), but the difference to controls did not reach a significant level. Furthermore, a lower percentage of non-atrophic gastritis patients reported lacking waste removal by garbage truck (10.6 %) as compared to patients with ChAG (18.8 %) and GC (26.4 %). But the difference was only significant for gastric cancer patients. In addition, more cases were found to live in rural areas or shantytown and boiled water non-standard means as compared to controls. Again, significant levels were reached only for gastric cancer cases. Education level and materials used for house construction were also different and are displayed in Table 7.14.

Table 7.14. Living conditions of all cases and controls (*n*=419) that were significantly different in an unconditional logistic regression model.

Characteristics	Controls (NAG)	Cases (ChAG)		Cases (GC)	
	n (%)	n (%)	*P**	n (%)	*P**
Education					
middle school	87 (45.5)	21 (42.9)	Ref.	77 (43.0)	Ref.
college and above	36 (18.8)	5 (10.2)	0.26	14 (7.8)	**0.012**
low education level	68 (35.6)	23 (46.9)	0.65	88 (49.2)	0.076
Home location					
Urban-Marginal	147 (83.1)	37 (77.1)	Ref.	105 (61.8)	Ref.
Rural	22 (12.4)	9 (18.8)	0.17	43 (25.3)	**0.001**
Shanty town	8 (4.5)	2 (4.2)	0.77	22 (12.9)	**0.004**
Material for house					
Cement	165 (86.4)	41 (85.4)	Ref.	129 (73.3)	Ref.
Corrugated plate/earth	26 (13.6)	7 (14.6)	0.63	47 (26.7)	**0.001**
Custody of food					
Refrigerator	156 (84.8)	35 (77.8)	Ref.	110 (64.3)	Ref.
No refrigerator	28 (15.2)	10 (22.2)	0.14	61 (35.7)	**0.0001**
Water preparation					
Everytime cooked	129 (69.0)	28 (62.2)	Ref.	91 (53.5)	Ref.
Not everytime cooked	58 (31.0)	17 (37.8)	0.5	79 (46.5)	**0.003**
Waste removal					
Garbage truck	168 (89.4)	39 (81.3)	Ref.	128 (73.6)	Ref.
No garbage truck	20 (10.6)	9 (18.8)	0.11	46 (26.4)	**0.0001**

Non-atrophic gastritis (NAG), chronic atrophic gastritis (ChAG), gastric gancer (GC)
*unconditional logistic regression model adjusted for age, sex and *H. pylori* infection

Since habit and living circumstances (environmental factors) were different for cases and controls (albeit only significant for gastric cancer), controls were frequency-matched to cases with respect to environmental factors, age (± 10 year), and gender. A matching of 1:1 using adequate controls was feasible for 133 cases with incident non-cardia gastric cancer. Furthermore, appropriate controls were available for 43 chronic atrophic gastritis cases to match 2:1 (control:cases). Finally, from all non-atrophic gastritis patients, 158 were usable for matching and no significant difference regarding environmental factors, age and sex were observed between matched groups.

A total of 334 patients (NAG, *n*=158; ChAG, *n*=43; GC, *n*=133) were subjected to statistical analysis regarding polymorphisms of IL-1B-511 and IL-1RN. Genotype frequencies in matched cases and controls did not deviate from the Hardy-Weinberg equilibrium (Table 7.15).

Table 7.15. Hardy-Weinberg Equilibrium (HWE) for matched cases and controls.

	HWE†						HWE¥					
	NAG (Controls)†			ChAG (Cases)†			NAG (Controls)¥			GC (Cases)¥		
Genotype	obs.[a]	(exp.)[b]	*P*[c]	obs.	(exp)	*P*	obs.	(exp.)	*P*	obs.	(exp.)	*P*
IL-1B-511												
C/C	3	(3.36)		7	(7.12)		7	(6.54)		15	(15.91)	
C/T	28	(27.28)		21	(20.76)		45	(45.91)		62	(60.18)	
T/T	55	(55.36)	1.0	15	(15.12)	1.0	81	(80.54)	0.8	56	(56.91)	0.85
IL-1RN												
*2/*2	10	(12.66)		8	(8.15)		16	(18.80)		16	(19.94)	
*2/L	46	(40.67)		21	(20.70)		68	(62.41)		71	(63.12)	
L/L	30	(32.66)	0.26	13	(13.15)	1.0	49	(51.80)	0.36	46	(49.94)	0.2

†Controls and cases are matched 2:1 for age, sex and environmental factors
¥Controls and cases are matched 1:1 for age, sex and environmental factors
[a]observed
[b]expected
[c]Fisher´s Exact test

7.3 INCREASED RISK FOR CHRONIC ATROPHIC GASTRITIS

For the IL-1B-511 SNP, 34.9 % of chronic atrophic gastritis patients (cases) and 64.0 % of patients with non-atrophic gastritis (controls) were homozygous carriers of the T allele (Table 7.16). Homozygous carriers of the C allele were very rare in controls at 3.5 %. In contrast to studies from Europe and North America, the T allele of the IL-1B-511 SNP was more common in controls (80 %). Due to that finding, the T allele served as the reference for the calculation of odds ratio (OR) in a conditional logistic regression model adjusted for *H. pylori* infection.

The C allele (C/T and C/C genotypes) of the IL-1B-511 SNP was associated with a significantly (*P*=0.001) higher risk for atrophic gastritis (OR, 5.60; 95 % CI, 2.02-15.51) when compared to non-atrophic gastritis patients (Table

7.16). Homozygous carriers of the IL-1B-511 C allele (C/C genotype) even showed a higher risk association (OR, 11.2; 95 % CI, 2.27-55.37) for this premalignant lesion than patients heterozygous for this allele (OR, 4.8; 95 % CI, 1.65-13.83).

Table 7.16. Homozygous and heterozygous patients with the IL-1B-511 C allele showed a significantly higher risk for chronic atrophic gastritis.

	NAG (Controls)	ChAG (Cases)		
Genotype	*n* (%)	*n* (%)	OR (95% CI)*	*P* *
IL-1B-511				
C/C	3 (3.5)	7 (16.3)	11.2 (2.27-55.37)	**0.003**
C/T	28 (32.6)	21 (48.8)	4.8 (1.65-13.83)	**0.004**
C carrier	31 (36.0)	28 (65.1)	5.6 (2.02-15.51)	**0.001**
T/T	55 (64.0)	15 (34.9)	1.0 (Reference)	
IL-1RN				
*2/*2	10 (11.6)	8 (19.0)	1.6 (0.48-5.39)	0.43
*2/L	46 (53.5)	21 (50.0)	1.0 (0.44-2.34)	0.98
*2 carrier	56 (65.1)	29 (69.0)	1.1 (0.50-2.48)	0.79
L/L	30 (34.9)	13 (31.0)	1.0 (Reference)	

*conditional logistic regression model adjusted for *H. pylori* infection
controls and cases are matched 2:1 for age, sex and environmental factors

For the IL-1RN, 4 genotypes (1/1, 1/2, 2/2 and 2/3) were found in the study population and alleles *1 and *3 (>2 repeats) were designated as L allele. In chronic atrophic gastritis patients and controls, the L allele was more common than the *2 allele (allele frequencies: L allele, 62 %; *2 allele, 38 %). The heterozygous *2 allele (2/L) was found in 50.0 % of the cases and in 53.5 % of the controls. The ratio of homozygous *2 allele carriers was found to be higher for cases (19.0 %) than for controls (11.6). However, for the IL-1RN polymorphism, all calculated *P* values were above the significant level of 0.05. There was no significant increased risk for chronic atrophic gastritis patients homozygous or heterozygous for the *2 allele as compared to patients who were homozygous for the L allele (OR, 1.6; 95 % CI, 0.48-5.39 and OR, 1.1; 95 % CI, 0.50-2.48, respectively) (Table 7.16).

It was also examined whether the frequency of the C allele has impact on the location of the chronic atrophic gastritis i.e. in the antrum or body (Table 7.17). In the antrum region there is a considerably higher risk for chronic atrophic gastritis in

patients bearing the IL-1B-511 C allele (OR, 4.0; 95 % CI, 1.63-9.81) as compared to the homozygous reference allele variant (genotype T/T). The highest risk for chronic atrophic gastritis was found for the homozygous C allele carriers (OR, 12.7).

Table 7.17. Location of diagnosis. For homozygous IL-1B-511 C allele carriers a 12-fold higher risk of getting chronic atrophic gastritis was found, but this association was restricted to the antrum.

	Location of diagnose			
	Antrum		**Corpus**	
	NAG[a]	ChAG[b]	NAG	ChAG
IL-1B-511 genotype				
C/C	2 (3.3)[c]	6 (16.7)	1 (4.0)	1 (14.3)
C/T	21 (34.4)	19 (52.8)	7 (28.0)	2 (28.6)
C carrier	23 (37.7)	25 (69.4)	8 (32.0)	3 (42.9)
T/T	38 (62.3)	11 (30.6)	17 (68.0)	4 (57.1)
C carrier vs. T/T				
OR (95% CI)*		4.0 (1.63-9.81)		1.5 (0.24-10.06)
*P**		**0.002**		0.65
C/C vs. T/T				
OR (95% CI)*		12.7 (2.12-76.43)		4.8 (0.07-360.6)
*P**		**0.005**		0.47

*unconditional logistic regression model adjusted for and *H. pylori* infection
[a]Controls
[b]Cases
[c]n (%)

7.4 INCREASED RISK FOR GASTRIC CANCER

Similar to chronic atrophic gastritis, association between the IL-1B-511 SNP and gastric cancer was found showing that the homozygous C allele variant of the IL-1B-511 SNP as well as the heterozygous allele variant were more frequent in patients with gastric cancer (OR, 4.15; 95 % CI, 1.33-12.93 and OR, 2.17; 95 % CI, 1.23-3.84, respectively) (Table 7.18).

Table 7.18. Genotype distribution for NAG and GC. The risk for gastric cancer was significantly increased for IL-1B-511 C carriers or for patients homozygous for the C allele.

	NAG (Controls)	GC (Cases)		
Genotype	*n* (%)	*n* (%)	OR (95% CI)*	*P* *
IL-1B-511				
C/C	7 (5.3)	15 (11.3)	4.15 (1.33-12.93)	**0.014**
C/T	45 (33.8)	62 (46.6)	2.17 (1.23-3.84)	**0.007**
C carrier	52 (39.1)	77 (57.9)	2.36 (1.36-4.11)	**0.002**
T/T	81 (60.9)	56 (42.1)	1.0 (Reference)	
IL-1RN				
*2/*2	16 (12.0)	16 (12.0)	0.86 (0.34-2.14)	0.75
*2/L	68 (51.1)	71 (53.4)	1.03 (0.59-1.80)	0.91
*2 carrier	84 (63.2)	87 (65.4)	0.99 (0.57-1.71)	0.99
L/L	49 (36.8)	46 (34.6)	1.0 (Reference)	

*conditional logistic regression model adjusted for *H. pylori* infection
controls and cases are matched 1:1 for age, sex and environmental factors

Cases and controls had similar frequency of homozygous *2 allele carriers (12.0 %), and no difference was observed between the two other genotype variants (*2/L and L/L). A significant association between gastric cancer risk and IL-1RN VNTR could not be reported in this study. For controls, the calculated allele frequency for both polymorphisms was as follows: 78 % for the IL-1B-511 T allele and 38 % for the IL-1RN *2 allele.

Further statistical analysis was conducted for the different histological type of gastric cancer (diffuse and intestinal gastric cancer) and location (antrum and corpus) to see whether there was a correlation with the distinct polymorphisms in the IL-1 gene cluster.

Stratification by location and histological type of gastric cancer showed that the C allele of the IL-1B-511 SNP was associated with a higher frequency of intestinal (OR, 2.6; 95 % CI, 1.0-6.5) but not with diffuse-type of gastric cancer (Table 5). The homozygous C allele variant showed a six-fold increased risk of intestinal gastric cancer located in the corpus of the stomach compared to non-atrophic gastritis controls (OR, 6.3; 95 % CI, 1.2-33.8) (Table 7.19).

Table 7.19. For homozygous IL-1B-511 C allele carriers a 6-fold higher risk of getting intestinal-type gastric cancer was found, but this association was restricted to the corpus region.

	Location of diagnose					
	Antrum			Corpus		
	NAG[a]	IT-GC[b]	DT-GC[c]	NAG	IT-GC	DT-GC
IL-1B-511 genotype						
C/C	5 (5.3)[d]	2 (4.0)	1 (5.0)	2 (5.3)	9 (21.4)	3 (14.3)
C/T	32 (33.7)	25 (50.0)	11 (55.0)	13 (34.2)	17 (40.5)	9 (42.9)
C carrier	37 (38.9)	27 (54.0)	12 (60.0)	15 (39.5)	26 (61.9)	12 (57.1)
T/T	58 (61.1)	23 (46.0)	8 (40.0)	23 (60.5)	16 (38.1)	9 (42.9)
C carrier vs. T/T						
OR (95% CI)*		1.9 (0.9-3.8)	2.4 (0.9-6.3)		2.6 (1.0-6.5)	2.3 (0.7-7.0)
*P**		0.087	0.089		**0.042**	0.155
C/C vs. T/T						
OR (95% CI)*		1.0 (0.2-5.8)	1.3 (0.1-13.2)		6.3 (1.2-33.8)	5.00 (0.7-37.3)
*P**		0.97	0.82		**0.032**	0.116

*unconditional logistic regression model adjusted for *H. pylori* infection
[a]Controls
[b]Cases (Intestinal-type gastric cancer)
[c]Cases (Diffuse-type gastric cancer)
[d]n (%)

Analysis for the IL-1RN polymorphisms did not show a pronounced increased risk of having either intestinal-type or diffuse-type gastric cancer in patients homozygous or heterozygous for the *2 allele as compared to patients who were homozygous for the L allele.

7.5 *H. PYLORI* INFECTION

The risk of intestinal-type gastric cancer and chronic atrophic gastritis related to IL-1B-511 genotypes was further examined with stratification by *H. pylori* infection. Analysis revealed a higher risk for chronic atrophic gastritis only for infected patients whereas patients homozygous for the C allele of the IL-1B-511 SNP had the highest risk compared to patients carrying the heterozygous C allele variant (OR, 10.17 and OR, 4.43, respectively) (Table 7.20).

Similar, only *H. pylori* infected patients with the IL-1B-511 C allele variant were under a higher risk for intestinal-type gastric cancer as compared to infected non-atrophic gastritis patients homozygous for the T allele. The highest risk for intestinal-type gastric cancer was found among homozygous C allele carriers (OR,

12.24), whereas patients heterozygous for the C allele of the IL-1B-511 SNP showed a lower risk (OR, 3.34).

Table 7.20. *H. pylori* infection and the IL-1B-511 SNP. A significantly higher risk for chronic atrophic gastritis or intestinal-type gastric cancer was observed for patients infected with *H. pylori* and bearing the C allele of the IL-1B-511 SNP.

	NAG[a]	ChAG[b]	NAG[a]	IT-GC[c]
IL-1B-511 genotype				
C/C	3 (3.9)[d]	5 (15.2)	2 (2.5)	8 (11.8)
C/T	25 (32.9)	18 (54.5)	21 (25.9)	31 (45.6)
C carrier	28 (36.8)	23 (69.7)	23 (28.4)	39 (57.4)
T/T	48 (63.2)	10 (30.3)	58 (71.6)	29 (42.6)
C carrier vs. T/T				
OR (95% CI)*		5.08 (1.95-13.21)		4.11 (1.91-8.84)
*P**		**0.001**		**0.0001**
C/T vs. T/T				
OR (95% CI)*		4.43 (1.65-11.91)		3.34 (1.51-7.35)
*P**		**0.003**		**0.003**
C/C vs. T/T				
OR (95% CI)*		10.17 (1.86-55.75)		12.24 (2.12-70.64)
*P**		**0.008**		**0.005**

*unconditional logistic regression model adjusted for age and sex

[a]*H. pylori* infected controls

[b]*H. pylori* infected chronic atrophic gastritis cases

[c]*H.pylori* infected cases with intestinal-type gastric cancer

[d]n (%)

Analysis for *H. pylori* negative patients did not reveal any increased risk for gastric cancer or chronic atrophic gastritis.

In addition it was questioned whether a distinct allele of the investigated polymorphisms is associated with higher risk for *H. pylori* infection. Analysis revealed similar distribution of genotypes for both polymorphisms (IL-1B-511 and IL-1RN VNTR) between *H. pylori* infected and non-infected controls (NAG).

8 DISCUSSION

8.1 STUDY'S MAJOR FINDING

Given a high load of infection by virulent (*cag*A positive, *vac*A toxigenic) *H. pylori* strains in Amerindian Peruvian populations [66], it seems likely that host genetic factors affect the risk of emergence and progression of premalignant lesions (chronic-atrophic gastritis) in *H. pylori*-infected Peruvians as in other populations.

In the Peruvian study population, a significant association was found between the IL-1B-511 SNP and increased risk for chronic atrophic gastritis as well as for gastric cancer. The heterozygous C allele variant of the IL-1B-511 SNP was associated with a higher risk for chronic atrophic gastritis (OR, 4.79) and gastric cancer (OR, 2.17) and highest risk for both pathologies was observed for the homozygous C allele variant (ChAG, OR, 11.22 and GC, OR, 4.15). Stratification by disease location revealed significant associations for ChAG in the antrum region and for intestinal-type gastric cancer in the corpus.

The findings of the present study confirm and extend the previous observation that SNPs as a host factor are involved in the susceptibility of intestinal-type gastric cancer and its precancerous lesion, e.g. atrophic gastritis, in *H. pylori*-infected patients. Furthermore, it suggests a different mechanism in the carcinogenesis of diffuse-type gastric cancer. In addition, this study is the first to verify the C allele of the IL-1B-511 SNP as a risk factor in a population from South-America.

8.2 STUDY DESIGN

A case-control study was used to compare exposure to genetic risk factors (IL-1B-511 SNP, IL-1RN VNTR) between non-atrophic gastritis patients as controls and individuals diagnosed with chronic atrophic gastritis or gastric cancer. Since *H. pylori* prevalence is very high in Peru [67], histological findings of healthy gastric mucosa are very rare and appropriate selection of controls was assumed by non-atrophic gastritis patients. The use of uninfected healthy controls would lead to an overestimation of the effect of genetic variants in the IL-1ß gene cluster due to the marked impact of *H. pylori* on gastric mucosal changes [68]. Cases of gastric cancer or ChAG represented a subset of all patients admitted to the affiliated hospitals and therefore controls were sampled from the same institution.

Recently it has been shown in a study from Peru, that *H. pylori* infection rate is linked to water supply, level of hygiene and sanitation especially among children [69] and also depends on socioeconomic status [8;10]. Assuming that patients differed with regards to their socioeconomic level and environmental factors, a 130-item questionnaire was achieved from every patient to test for homogeneity in cases and controls. In the present study environmental factors showed a significant association with gastric cancer risk. Especially the lack of regular waste removal was more common for gastric cancer cases than for non-atrophic gastritis patients. In addition, more cases had no refrigerator and did not boil water in a standard fashion.

This suggests that the outcome of a *H. pylori* infection is modulated in part by living circumstances and habits of the infected host. Therefore, cases and controls were matched to yield the required homogeneity in the study population. Homogenous genetic background between patients from both municipal hospitals was assumed since all individuals were Mestizos.

8.3 ALLELE FREQUENCIES

Comparison of the T allele frequency in healthy and non-atrophic gastritis patients from different studies (Figure 2.1) showed that the T allele of the IL-1B-511 SNP is more frequent in Asians and South Americans (45 % - 55 %). The present study observed an even higher frequency of around 80 % in non-atrophic gastritis patients. Due to that finding, the T allele served as a reference allele variant for subsequent statistical analysis. To date, this is the highest reported frequency for the IL-1B-511 T allele. RFLP technique was used for genotyping and the accuracy of assay was controlled by reanalyzing one fifth of samples from the preceding setting in the subsequent run. Furthermore, several samples were sequenced for the SNP and for each run all genotype variants (C/C, C/T, T/T) were included as positive controls. Since genotypes of all reanalyzed samples were concordant, a proper working assay can be assumed.

The *2 allele of the IL-1RN VNTR showed higher frequency (38 %) in comparison to reports from Asia but similar to the studies from Europe (22 % - 46 %). Taken together, the allele frequencies of the IL-1RN VNTR were comparable to European populations whereas the IL-1B-511 SNP alleles showed frequencies more similar to that of Asian populations. Reported allele frequencies from the present study are interesting, since the study's population consisted of Mestizos, a mixed population of Amerindians and Europeans, whereas Amerindians have ancestors from Central Asia.

8.4 POLYMORPHISMS IN THE IL-1B GENE CLUSTER

It has recently been suggested that interaction between bacterial virulence factors and immune response of the host affect the outcome of *H. pylori* infection and may be influenced by polymorphisms in both bacterium and host [36;70]. Especially polymorphisms in IL-1B and IL-1RN genes were found to be associated with a higher risk for gastric cancer development due to increased IL-1ß level in gastric mucosa in response to *H. pylori* infection [36;71]. El-Omar *et al.* described SNPs in

the IL-1B gene at positions -511, -31, +3954 bp from the transcriptional start site and showed significant associations to *H. pylori*-associated gastric cancer only for the first two positions [26]. Since these two regions are in total linkage disequilibrium, this thesis focused on the IL-1B-511 SNP.

8.4.1 HIGHER RISK FOR CHAG AND GC

The present study revealed a significant association between the IL-1B-511 C allele gastric cancer and its premalignant lesion, chronic atrophic gastritis. This is in line with studies from Japan and China that reported a similar association for the C allele [44;48] and that the C/C genotype is more frequent in low acid producers [63]. Inflammation in the stomach induced by *H. pylori* infection correlates with high IL-1ß level [72] which triggers immune response. Furthermore, enhanced IL-1ß level in gastric mucosa continuously increases the pH by inhibiting gastric acid production in the stomach, paving the way for progressive mucosal changes with a subsequent increased risk for gastric carcinogenesis.

Since chronic atrophic gastritis was associated with the IL-1B-511 C allele in the present study, Peruvians bearing the C allele might have high mucosal IL-1ß levels resulting in lower gastric acid output (hypochlorhydria). This has to be confirmed in further studies by measuring gastric output and mRNA of IL-1ß in non-atrophic gastritis patients.

The heterozygous genotype (C/T) showed a significant association with chronic atrophic gastritis as well as gastric cancer (OR, 4.79 and 2.17, respectively). Homozygosity for the C allele of the IL-1B-511 SNP was even more strongly associated with both pathologies, ChAG (OR, 11.2) and GC (OR, 4.15). The increasing odds ratio from heterozygous to homozygous carriers of the C allele suggests an additive effect of this polymorphism on the IL-1ß level.

These findings differ from those of previous studies in which the opposite allele (T allele) of the IL-1B-511 SNP was noted as a risk factor for chronic atrophic gastritis and gastric cancer [26;29;36;37]. However, most of these studies were conducted in Caucasian populations that showed a lower T allele frequency (<38 %) than frequencies found for Asians (45-55 %). In the Peruvian population, the T allele frequency was observed to be around 80 % which is more similar to that of Asians. That may in part be due the Amerindians with origins in Central

Asia, still reflecting the genetic background [9]. The even higher frequency of the T allele in comparison to Asians might be due to selective pressure on genotypes of Peruvian population.

8.4.2 LOCATION OF DIAGNOSIS AND GASTRIC CANCER SUBTYPES

Given the fact that antrum and corpus region differ in their function and cell composition, it was further investigated whether there is a marked difference in the effect of the C allele on gastric cancer subtypes and location of histological diagnosis. Indeed, a higher risk was found for intestinal-type gastric cancer in corpus (OR 6.29) for patients bearing the homozygous IL-1B-511 C allele variant. In addition, chronic-atrophic gastritis was more frequent in antral gastric biopsies and only in this region was a higher risk for atrophy associated with the IL-1B-511 C allele (OR 12.72).

One explanation could be the low prevalence of atrophy detected in the corpus, but, on the other hand, the *H. pylori* infection might start in the antrum or be restricted to the antrum due to intact corpus mucosa characterized by normal gastric acid output. The observed higher frequency of antrum atrophic gastritis has also been reported by previous studies from Peru [67;73;74].

Specialized cells of the stomach mucosa control gastric acid stimulation and secretion. While the hormone gastrin is released in the antrum region by G cells, enterochromaffine-like cells (ECL) in the corpus region respond with cell growth and the release of histamine [75;76]. Histamine from ECL cells together with gastrin from G cells stimulates parietal cells in the corpus to secrete gastric acid [77]. Deregulation can occur in this circuit if stomach is infected with *H. pylori* whereas alteration of acid secretion also depends on affected cells. Mostly, the inflammatory process starts in the antrum and affects inhibitory somatostatin cells, leading to higher gastrin levels and increased acid secretion in the corpus. High acid secretion hinders *H. pylori* colonization of the corpus and restricts the inflammation to the antral region. This pattern is also seen in duodenal ulcers, where a healthy corpus continues to secret gastric acid which is responsible for the epithelial damage in the duodenum. If *H. pylori* gastritis affects the corpus

region with its parietal cells, gastric secretion is decreased, which leads to higher pH. These are excellent conditions for further colonization of *H. pylori* and maintenance of the infection and can subsequently lead to atrophy in the corpus.

Once *H. pylori* gastritis affects the corpus region, inflammatory cytokines (IL-1ß and TNFα) can inhibit acid-secreting parietal cells, leading to a reduced gastric acid secretion [28]. The C allele may lead to an even higher IL-1ß level and subsequent enhanced acid reduction.

Gastric mucosa attempts to adjust the decreased acid output by enhanced gastrin secretion that stimulates parietal cells. Since the corpus is inflamed and parietal cell function is decreased, acid output remains low, leading to pronounced atrophy in the corpus. Sustained inflammation and high gastrin levels act as a proliferative stimulus on epithelial cell [27] and, in combination with reactive oxygen species (ROS), bacterial overgrowth, and nitrosamines, lead to an increased risk of DNA damage, mutagenesis and progression to carcinogenesis.

This is confirmed by the present finding that patients with the C allele were under significantly higher risk for intestinal-type gastric cancer only in the corpus. The different impact of the IL-1B-511 SNP to the IL-1ß level in antrum and corpus in response to *H. pylori* infection has to be considered as an underlying mechanism. This is supported by a previous study showing higher mucosal IL-1ß levels in the gastric body than in the antrum region of patients with the IL-1B -511 SNP C allele [72].

In this regard, the atrophy in the antrum could be interpreted as a first step in a multistage progression that can lead to *H. pylori* colonization of the corpus as well, along with an increased risk of intestinal-type gastric cancer in patients bearing the IL-1B-511 C allele.

8.4.3 THE IL-1RN VNTR

The second investigated host gene, IL-1RN, encodes for the IL-1ra protein, showing the same affinity as IL-1ß to the corresponding receptor (IL-1R). Since IL-1ra does not induce any intracellular response, it is acting in a competitive manner with respect to IL-1ß and thereby subsequently modulates the proinflammatory effects of IL-1ß [32].

The *2 allele of the VNTR in the intron 2 has been linked to higher IL-1ß production in vitro [33] and seems to be associated with atrophic gastritis [36] and early-stage gastric cancer [78].

The present study has not revealed any significant association between the IL-1RN VNTR and atrophic gastritis, similar to several studies from Asia. In contrast, especially studies with Caucasian populations have reported a significant association between the IL-1RN *2 allele and outcome of *H. pylori* infection (i.e. chronic atrophic gastritis, gastric cancer). The difference between the present findings and those from Europe might depend on the regulative role of IL-1ra to IL-1ß. IL-1ra and IL-1ß might act hand in hand, so that increasing IL-1ra level would provoke an enhanced IL-1ß level to maintain a given IL-1ß/IL-1ra ratio. Virulence factors of *H. pylori* along with the SNP at IL-1B-511 may cause such a high basal IL-1ß level and/or prolonged effects of IL-1ß that diminish the importance of IL-1ra variants as regulators of the IL-1ß synthesis in the Peruvian population. Furthermore, if the 3 different genotypes (*2/*2, *2/L, L/L) in Peruvians cause same IL-1ra protein level, no alteration in the IL-1ß level would succeed and might also explain the lacking association.

Taken together, the above-mentioned findings suggest that this distinct SNP and its association with a higher risk for ChAG and GC is population-specific and depends on the allele frequency in the study population.

8.5 *H. PYLORI* INFECTION

H. pylori infected and non-infected controls (NAG) showed similar distribution of genotypes for both polymorphisms (IL-1B-511 and IL-1RN VNTR). Thus, neither the IL-1B SNP nor the IL-1RN VNTR were associated with a higher risk for *H. pylori* infection, which itself is a risk factor for chronic atrophic gastritis and gastric cancer [68]. This suggests that the observed association between the IL-1B SNP and higher risk for both gastric diseases are caused by the impact of the C allele rather than by increased susceptibility for *H. pylori*.

Blaser and Atherton [79] proposed a mathematical model that describes the equilibrium between colonized bacterium and gastric mucosa of the host whereas cross talk occurs by direct contact or soluble mediators. When activation of the host's immune response disturbs equilibrium, *H. pylori* infection may persist

due to selective mechanism for the fittest bacterium or due to altered protein secretion by the bacterium itself. Achieving a new equilibrium level maintains the chronic bacterial infection without impairment of colonization conditions for *H. pylori* in the gastric mucosa. This assumption is supported by epidemiological studies from Peru where about one-half of Peruvian children become infected with *H. pylori* during their first years, and most others become infected within the following next one to two decades [18;19], suggesting a lifelong infection in this population.

The proportion of *H. pylori*-positive patients was highest in the non-atrophic gastritis group (88 %), reflecting the high prevalence of this bacterial infection in Peruvian gastritis patients. These findings are in line with previous reports from Peru claiming that nearly 90 % of adults undergoing upper-gastrointestinal endoscopy due to dyspeptic symptoms were found to be infected with this bacterium [14;15]. The lower rate of *H. pylori* infection was revealed for the chronic atrophic gastritis patients (79.6 %) and for gastric cancer group (74.2 %). Since atrophic gastritis or cancerous mucosa is unsuitable for *H. pylori* colonization [23], it is possible that some *H. pylori*-negative cases had previously been infected.

However, the observed decrease in *H. pylori* prevalence in ChAG and GC underlines the fact that worsen mucosal conditions cause disbalance between a host's gastric mucosa and bacteria. *H. pylori* itself and an atrophic mucosal environment can lead to DNA damage in gastric epithelial cells [80], optimal conditions for carcinogenic process.

8.6 CONCLUSION

To summarize, the following conclusion can be drawn based on previous and present findings:

H. pylori infection in combination with a functional IL-1B variant can lead to increased IL-1ß secretion and subsequent decline of gastric acid secretion. Restriction of the bacterial infection to the antrum region is associated with normal gastric acid output but higher a risk for antral atrophic gastritis. Once *H. pylori* infection encroaches upon the corpus region, inflammation subsequently leads to higher risk for intestinal-type gastric cancer.

This study is the first to establish the C allele of the IL-1B-511 SNP as a risk factor for intestinal-type gastric cancer and its premalignant lesion gastric atrophy in a largely Amerindian population. These findings are consistent with the progression of histological changes from chronic atrophic gastritis to intestinal-type gastric cancer. Furthermore, it suggests differences in processes leading to diffuse-type gastric cancer.

In conjunction with previous studies, our results show the importance of population-based SNP investigations regarding gastric cancer risk. Our findings support the hypothesis that inter-individual, genetically determined differences of cytokine production affect outcomes of *H. pylori* infection and gastric cancer risk. In view of a high *H. pylori* prevalence and a high recurrence rate of treated infection in Peruvians with low socioeconomic status [20], IL-1B-511 SNP might be an indicator for patients who will benefit from rigorous anti *H. pylori* therapy and intensive surveillance of gastric mucosal changes by means of intermittent gastroscopies.

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10 DECLARATION

Ich erkläre, dass die der Medizinischen Fakultät der Otto-von-Guericke-Universität zur Promotion eingereichte Dissertation mit dem Titel

“The C allele of the IL-1B-511 SNP is associated with higher risk for Gastric Cancer and its premalignant lesion:

A prospective case-control study in Peru“

selbstständig, ohne fremde Hilfe und ohne Benutzung anderer als der angegebenen Quellen und Hilfsmittel von mir angefertigt wurde. Alle Ausführungen, die wörtlich oder sinngemäß übernommen wurden, sind als solche gekennzeichnet.

Bei der Abfassung der Dissertation sind Rechte Dritter nicht verletzt worden. Diese Arbeit ist oder war nicht Gegenstand eines anderen Prüfungs- oder Promotionsverfahrens. Ich habe noch keinen Doktorgrad erlangt oder zu erlangen versucht. Ich übertrage der Medizinischen Fakultät das Recht, weitere Kopien meiner Dissertation herzustellen und zu vertreiben.

Magdeburg, den 23.04.2008

11 ACKNOWLEDGEMENTS

First of all, I would like to gratefully acknowledge Prof. Robert Gilman for the opportunity to work in his laboratory at the Universidad Peruana Cayetano Heredia. He provided my research project, guided me during my stay in Lima and gave me support throughout. I am glad that I was able to spend 6 months with such an advisor and colleagues who always taught me innumerable lessons and insights on academic research. Prof. Gilman and PRISMA were considerably responsible for the good conditions of this research project.

In addition, I would like to express sincere appreciation to Dr. Meyer.

Despite the distance, he gave me invaluable advice and was always available when I needed him. He helped me substantially during the preparation of this thesis. His editorial advice was essential to the completion of this dissertation. Thank you for all your support!

My cordial thanks go to Prof. Dr. Lippert for critically reviewing the thesis and assistance regarding all aspects.

My warmest regards to the research team of Prof. Robert Gilman, especially Billie Velapatiño, Paula Maguina, Phabiola Herrera, Jaquiline Balqui, Livia Santivañez, Vilma Gutierre, Jonathan Sherman, Vivi Cancino, Mrs. Carmen, Vanessa Chung, Daniel Velasquez and all other co-workers for their daily co-operation and the pleasant atmosphere, as well as their help with all of the techniques which were intrinsic to the completion of the project.

I also want to acknowledge Dr. Jaemi Cok for the results of the histological investigation, which he completed alongside his daily work as a pathologist at the Hospital Nacional Cayetano Heredia.

Thank you to Sijin Wen, Department of Biostatistics at the MD Anderson Cancer Center Houston, USA for assistance with statistical analysis.

Special thanks to all my friends who have been very supportive during the entire period of my study.

I would also like to take this opportunity to express my deepest gratitude and warmest regards for my parents, my brother and my wife, Sanga. Their constant patience, support and encouragement made it possible to bring this thesis to an end. Thank you for always being there for me, looking out for my best and backing me up.

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