Non-Hodgkin's lymphoma of the gastro-intestinal tract : clinicopathological correlations

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Non-Hodgkin's lymphoma of the gastro-intestinal tract

Clinicopathological correlations

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Non-Hodgkin's lymphoma of the gastro-intestinal tract

Clinicopathological correlations

PROEFSCHRIFT

ter verkrijging van de graad van doctor
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Voor Pap en mam
Voor mijn oom Piet⁺

Contents

Chapter 1	General Introduction	9
Chapter 2	Materials and Methods	43
Chapter 3	Proliferation and apoptosis in primary gastric B-cell non-Hodgkin's lymphoma	57
Chapter 4	Specific detection of <i>Helicobacter pylori</i> and non- <i>Helicobacter pylori</i> flora in small- and large-cell primary gastric B-cell non-Hodgkin's lymphoma	71
Chapter 5	<i>Helicobacter pylori</i> and non- <i>Helicobacter pylori</i> bacterial flora in gastric mucosal and tumour specimens of patients with primary gastric lymphoma	81
Chapter 6	In situ hybridisation studies in small and large cell gastric MALT lymphoma demonstrate higher numbers of numerical chromosomal aberrations in large cell lymphomas	97
Chapter 7	Retrospective survival analysis in gastric lymphoma reveals surgical therapy and MALT histology as favourable prognostic factors	109
Chapter 8	Cell turnover parameters in primary intestinal non-Hodgkin's lymphoma related to small and large cell varieties	123
Chapter 9	Primary intestinal non-Hodgkin's lymphoma: clinical description of 39 consecutive cases and correlations with cell turnover parameters	137
Chapter 10	General discussion	153
	Summary	159
	Samenvatting	165
	Appendix	171
	Dankwoord	177
	Curriculum vitae	181

Chapter 1

General Introduction

"The existence of deeply held divergent views concerning the histological identification of tumour cell types is a particularly serious obstacle, since the international adoption of a soundly based histopathological classification is a prerequisite to substantial progress at the clinical level."

(Kaplan, 1975)

General Introduction

Definition and classification of primary GI NHL: historical aspect

Primary gastro-intestinal (pGI) non-Hodgkin's lymphomas (NHL) by definition are NHLs which have their origin inside the gastro-intestinal tract. As simple this definition may seem, the harder it is to understand the problems which have been encountered in trying to define and classify these pGI NHL.

Few neoplastic diseases have been renamed and reclassified so often as the lymphoproliferative disorders¹⁻¹¹. Developments in immunohistochemical and molecular biological techniques have lead to an enormous progress in the understanding of the pathogenesis of this very diverse group of lesions. As a result classification systems have become more and more refined, combining pathological, biological and clinical data to well-defined entities. However, the definition and classification of the almost 40% of lymphomas originating outside the major lymphoid tissues has been less clarified. The first complicating factor is the use of the expression extranodal, which certainly excludes the lymph nodes, but in most cases also the spleen. Should the gastro-intestinal tract, which contains a considerable amount of lymphoid tissue be called extranodal¹²? Another problem is the definition of *primary* extranodal, because primary nodal NHLs often involve extranodal tissues as well. In the history of gastrointestinal lymphomas, these definition and classification problems take a central position.

The first reports of gastrointestinal lymphoproliferative lesions

In 1863, Virchow first recognized that malignant lymphoma sometimes occurs extranodally¹³, and in 1885, Hulbert reported about intestinal lymphoproliferative disorders¹⁴. Since these first publications a variety of reports on gastrointestinal lymphosarcoma followed but because the frequency of gastrointestinal lymphoma is relatively low, it was difficult to draw conclusions from these case reports. This was noticed by Crowther and his colleagues in 1913 and they collected the results of most evaluable cases reported until then into one study¹⁵. A few years later (1919) Graves analyzed the same cases supplemented with the data from case reports since 1913¹⁶.

In 1932 Ullmann and Abeshouse finished this extended case analysis using the cases of Crowther and Graves adding 126 cases to a total of 375 intestinal lymphosarcomas¹⁷. They concluded that the terms lymphosarcoma, lymphocytoma, lymphoblastoma, intestinal Hodgkin's disease, chronic inflammatory tumour, lymphoid granulation tumour and granulomatous pseudoleukemia had all been used interchangeably. These and other findings lead them to conclude that "the literature concerning the subject of lymphosarcoma of the intestines reveals a marked

diversity of opinions concerning its morphology, histogenesis and etiology. This state of affairs is further complicated by the lack of any uniform nomenclature". They advocated to adhere to the term lymphosarcoma at least until the histogenetical and morphological characteristics and relationships of the structural components of these tumours became more clearly established.

Thanks to the collections of separate cases of gastrointestinal lymphoproliferative lesions, interest in these neoplasms increased. They became recognized as a separate entity which needed further investigation to elucidate its nature. Emphasis was first put on the differentiation between lymphosarcoma and pseudolymphoma

Starting around the 1940s, various groups began to notice that true lymphosarcoma of the stomach in some cases could only be separated with difficulty from granulomatous pseudosarcoma or infectious granuloma and they concluded that the presence of plasma cells, eosinophils and normal lymphocytes suggested an inflammatory lesion^{18,19}. Other histopathological features that made the diagnosis of lymphoma doubtful were fibrosis and the presence of a granulomatous element²⁰.

In 1958 Smith and Helwig²¹ recognized that some lesions previously diagnosed as gastric lymphoma in fact were reactive lymphoid hyperplasia and few years later Helwig set up criteria for reactive lymphoid hyperplasia, which were presented in May, 1960 on a meeting of the Indiana Association of Pathologists: "the presence of a mixed inflammatory infiltrate with reaction centres and ubiquitous scar tissue intimately mixed in a lymphoreticular mass". Jacobs in 1963 studied 27 cases of gastric lymphoreticular proliferative disorders using these criteria and concluded that they satisfactorily distinguished between true neoplasms and benign lymphoreticular hyperplastic states which mimic them²².

One year later Faris and Saltzstein suggested that the better prognosis in patients with gastric lymphosarcoma could be due to the inclusion of patients with gastric lymphoid hyperplasia -which they believed was a non-neoplastic late stage of ulceration of the stomach (supported by the ulcer-like symptoms and the histological findings of longstanding ulceration)- in population samples of gastric lymphosarcoma²³. This was earlier suggested by Smith and Helwig, and later by Johnson and Sowerbutts²⁴ who presented a case report of a pseudolymphoma of the stomach to illustrate that pseudolymphoma accounted for some unexpected long-term survivors in patients originally diagnosed as suffering from malignant lymphoma.

Azzopardi²⁵ who found a surprisingly good prognosis in some large inoperable GI tumours, suggested that a possible reason for this could be the fact that primary GI lesions were more often localized and not disseminated at presentation.

On the basis of the criteria of Smith and Helwig combined with evidence of longstanding peptic ulceration (by Faris & Saltzstein) Berry and Matthews restudied 12 cases of gastric lymphosarcomas and found that six after restudy turned out to

be pseudolymphoma. They found a polymorphic cellular infiltrate, associated with a fibrotic reaction in the submucosa, the muscularis or the serosa to be the most helpful criterion²⁶. Perez and Dorfman studied benign lymphoid hyperplasia and found that pathological diagnosis is usually clear cut in the presence of an inflammatory infiltrate which includes numerous mature lymphocytes, plasma cells, eosinophils, and true lymphoid germinal centres, without evidence of regional lymph node involvement, in a patient with chronic peptic ulceration²⁷.

In 1964 Kay and colleagues also made an attempt to set up standards to differentiate between true neoplasms and tumour-like inflammatory reactions²⁸. They concluded that, in general, a true tumour will consist of aggregates of 'pure' lymphocytes with little or no other cellular elements, such as plasma cells, Russell bodies, polymorphonuclear leucocytes -except at the ulcerating surface- and macrophages.

Moreover, malignant tumours contained a solid mass of cells which in the more advanced stage infiltrated all layers of the stomach wall. Sometimes the presence of a row of follicles within the collections of lymphocytes was a prominent feature. They admitted that differentiation between a benign or a malignant lesion may at some times be impossible and also stated that a malignant lymphoma without regional lymph node involvement adequately resected should have a good prognosis, not necessarily implying a wrong diagnosis.

Anderson and colleagues also concluded that the differentiation in some cases can be almost impossible. In particular, when inflammation occurs in a malignant lymphoma, it can produce true lymphoid follicles with germinal centres²⁹. However, they stated that recognition of this benign condition is important to prevent unnecessary treatment and to prevent inclusion in series of malignant gastric lymphoma, in order not to positively influence (bias) high survival rates. Highman³⁰ reviewed the literature and also concluded that pseudolymphoma is a benign condition with an excellent prognosis, which, however, in some cases may be very difficult to distinguish from malignant lymphoma.

Others even distinguished different clinicopathological groups in benign lymphoid hyperplasia³¹: focal lymphoid hyperplasia of the stomach, focal lymphoid hyperplasia of the small intestine, focal lymphoid hyperplasia of the rectum and nodular lymphoid hyperplasia of the gastro-intestinal tract. They stated that lymphoid hyperplasia of the gastro-intestinal tract can be separated from malignant lymphoma by the polymorphic nature of the infiltrate, the absence of significant cytological atypia, and the presence of reactive follicles within the lesion.

In spite of general agreement that gastrointestinal lymphoproliferative lesions occurred in two manifestations, lymphosarcoma and pseudolymphoma, the difficulty or even inability to distinguish between them made some investigators consider that there could be a gradient from benign to malignant lymphoproliferative disease.

In 1966 Valdés-Dapena and his group studied histological features of gastric lymphoproliferative lesions and found a quantitative gradient in the extent of lymphoid hyperplasia, which made them speculate about a pathogenetic link between hyperplasia and neoplasia³². They hypothesized that causative factors of chronic gastritis may stimulate the lymphoid elements of the mucosa to sustained active proliferation, similar to the effect of the constant regenerative demands operating in colonmucosa in a condition like ulcerative colitis, which had been pathogenetically linked to carcinoma. Apart from this hypothetical link, it was obvious that a continuum existed extending from exuberant but benign lymphoid hyperplasia to highly infiltrating reticulum cell sarcoma. This was also described by Kahn and Novis who studied the association of polypoid nodular lymphoid hyperplasia (PNLH) of the small bowel associated with primary small bowel reticulum cell lymphoma³³. They believed that, although the association between PNLH and lymphoma could be fortuitous, the possibility that lymphoma evolves from hyperplasia should certainly be considered.

In 1981 Wolf reviewed the discussion about a pathogenetic link between hyperplasia and neoplastic development³⁴. Most authors at that time doubted about the existence of a consistent link between these two conditions, but some believed that giant follicular lymphoma of the stomach, a very rare entity, could represent a transition from benign hyperplasia to stomach lymphoma^{18,35,36}.

Causey hypothesized that hyperplasia and neoplasia could represent divers outlets of a common sustained immunologic stimulus³⁷. In this light, the two hit hypothesis described by Salmon and Seligmann could be important: a sustained immunologic stimulus can provoke a initial monoclonal expansion, which may set the stage for a subsequent transforming event³⁸.

Burke and colleagues used the term small lymphocytic proliferation to describe a lesion with the same histopathological features as those found in gastric pseudolymphoma³⁹. At the initial description of gastric pseudolymphoma it was considered to be a reactive lesion, and thus the infiltrate was assumed to be polyclonal. However Isaacson et al. have found that parts of these lesions were monoclonal and therefore, it could be a neoplastic lesion⁴⁰.

Sigal et al. investigated a case of a gastric lymphoproliferative disorder and found that the germinal centres and the immediately surrounding lymphocytes were polyclonal. However, the expanded areas of small, slightly irregular lymphoid cells between follicles expressed only IgM heavy chains and λ light chains. They believed that gastric pseudolymphoma and small lymphocytic lesions should be considered as lesions which have an increased risk of malignant transformation to full-blown malignant lymphoma. They hypothesized the following mechanisms underlying this chain of events⁴¹: The initiating event of many gastric pseudolymphomas could be mucosal ulceration with subsequent chronic antigenic stimulation leading to a dense, polyclonal lymphoid infiltrate. If continued antigenic

stimulation and proliferation occurred, one or more clones might emerge from the initial polyclonal response. Initially, proliferation of the (selected) clone might be dependent on antigenic factors, and thus at least under limited control, but with time the clonal proliferation might become autonomous, although this point could not be recognized morphologically. When the lesion progresses, the probability increases for cellular and architectural changes that may lead to morphologically recognizable lymphoma.

In the mean time, it became clear that it was very important to strictly define primary gastrointestinal lesions, since generalized lymphoproliferative disease often involved the gastrointestinal tract as well. Furthermore, in this rather rare condition, it was important to have the possibility to compare different studies, which was another reason for strict definitions.

In 1961 Dawson, Cornes and Morson⁴² pointed out some problems in the study of primary intestinal lymphoid tumours. First of all, that part of the reported cases were not unequivocally primary tumours of the intestines, secondly, that sometimes no refinements were given besides the term lymphosarcoma and thirdly that some benign lesions were not recognized as such. They described 37 cases, using the following strict criteria to consider these tumours primary:

1. no palpable superficial lymphadenopathy at first presentation,
2. no obvious enlargement of mediastinal lymph nodes,
3. normal white-blood cell-counts,
4. predomination of the bowel lesion at laparotomy, the only lymph nodes obviously affected being those in the immediate neighbourhood, and
5. no involvement of the liver and spleen.

Later studies showed that although gastrointestinal lymphomas have a tendency to remain localized, cases presenting with disseminated disease do exist, hence these criteria may be too strict.

With the development of immunohistochemistry it became possible to identify and classify the cell of origin in malignant lymphomas, which was often impossible on morphological grounds only. Primary gastrointestinal lymphoid tumours at that time were thought to be derived from either plasma cells or histiocytic cells, and investigations aimed at elucidating this question of the cell of origin using immunohistochemistry and other techniques.

Henry and Farrer-Brown⁴³ who had reviewed the histology of 125 cases of primary GI lymphoma using electronmicroscopy had found that 39% of their cases showed plasma cell tumour features.

In 1980 Yamanaka et al., studied the surface markers in gastrointestinal non-Hodgkin's lymphoma using specific antisera against human B-cells or T-cells⁴⁴. They found that the GI lymphomas they studied were derived from monoclonal

proliferations of malignant B lymphocytes in the digestive tract. In contrast, reactive lymphoid hyperplasia was polyclonal. The same conclusions were drawn by Grody et al.⁴⁵, who especially studied the large cell type lymphomas, which were often thought to be of histiocytic origin and demonstrated them to stain positive for either B- or T-cell markers, indicating their lymphatic derivation. Grody in this study used frozen material for immunohistochemistry to rule out the possible effects of formalin-fixation and found 84% of the studied cases to be of B-cell origin whereas none appeared to be histiocytic. Earlier studies by Otto et al.⁴⁶, MacLennan et al.⁴⁷, and Yamanaka et al.⁴⁴ also showed that GI NHL were primarily of B-cell type and rarely if ever of true histiocytic type. In contrast, Isaacson et al. who also used immunological markers for histocytes (α 1-antitrypsin and lysozyme) and lymphocytes (immunoglobulin heavy and light chains) reported that lymphomas of true histiocytic origin were especially frequent among primary lymphomas of the GI tract (50% of 66 cases examined)⁴⁸.

The first functional classifications for non-Hodgkin's lymphomas, like the Kiel classification and the classification of Lukes and Collins were also generated after the development of immunohistochemical techniques. However, these classifications were designed for nodal lymphomas and the question now arose how gastrointestinal lymphomas fitted in these 'nodal' classifications

Lewin, Ranchod and Dorfman⁴⁹ investigated whether extranodal lymphomas differ morphologically from nodal lymphomas or are similar to lymphomas encountered in lymph nodes. They used Rappaport's classification expanded to include the category of Mediterranean-type lymphoma (MTL). They classified only 14% as MTL, the histologic types of the remaining lymphomas were similar to those encountered in lymph nodes.

After its introduction in the seventies the Kiel classification became very popular as the first lymphoma classification referring to the cell of origin. Moreover, the classification proved to be useful in predicting prognosis. The use of the Kiel classification in gastric lymphomas and pseudo lymphomas was also studied. After the general consensus that the overwhelming majority of all malignant gastrointestinal lymphomas were derived from the lymphoid cell line, it was considered that most of the gastric lymphomas could be classified as immunocytoma, centroblastic/centrocytic, centroblastic or immunoblastic lymphoma⁵⁰. The diagnosis pseudolymphoma was made when a pleomorphic benign lymphoid population with predominance of small lymphocytes plus lymphoid follicles of various size with active germinal centres were seen.

Furthermore, all cases of pseudolymphoma were seen in the context of a follicular gastritis and the authors hypothesized that follicular gastritis, pseudolymphoma and primary malignant lymphoma might be related. The heavy lymphoid infiltration undoubtedly attested the existence of a prolonged antigenic stimulation which could

perhaps induce the development of a malignant clone. This had been earlier described by Dutz who examined the non-lymphomatous mucosa adjacent to primary malignant lymphoma of the stomach, and consistently found an associated perifoveal plasma cell gastritis which was absent in secondary lymphomas. Therefore, he believed the gastritis to be a precursor rather than a reaction to the lymphoma⁵¹.

In 1981 Isaacson⁵² dealt with the question how to fit in primary gastro-intestinal lymphomas in the new lymphoma classifications (Kiel classification, Lukes and Collins classification). He reported considerable agreement on the nature of many GI NHL, but controversy surrounding the nature of the lymphomas composed of larger cells: the 'histiocytic' lymphomas of Rappaport. At that time Isaacson thought that the discussion on histiocytic (i.e. monocyte/macrophage) derived tumours of the gastrointestinal tract remained.

The MALT concept

In the beginning of the eighties, a lot of controversies on the subject of gastrointestinal lymphomas were resolved. With the use of immunohistochemistry it was possible to distinguish in most cases between benign lesions and monoclonal malignant lesions, which made the use of the term pseudolymphoma unnecessary. Yet, questions about the (inflammatory ?) origin and (indolent ?) behaviour of these tumours remained.

In this period, Isaacson and Wright noticed a striking resemblance of gastrointestinal lymphomas with Mediterranean lymphomas, which made them propose the MALT concept: tumours of Mucosa-Associated Lymphoid Tissue.

In 1983 Isaacson and Wright first defined malignant lymphomas of mucosa-associated lymphoid tissue, after the discovery that some gastro-intestinal lymphomas shared certain histologic features with Mediterranean lymphomas (MTL)⁵³. The following features were described: "The superficial part of the mucosa is filled out with plasma cells, which can displace but do not invade the mucosal glands. In the deeper part the nature of the infiltrate changes and consists of a mixture of follicle centre cells, with scattered plasma cells among them. A characteristic invasion of individual glands by infiltrates of follicle centre cells (FCC) can be found. This invasion of individual glands serves as a useful feature to differentiate from reactive lymphoid infiltrates (pseudolymphoma)".

About the pathogenesis they speculated that the FCC's of gastrointestinal mucosal lymphoid nodules migrate into the lymphatics and enter the systemic circulation. The cells then home to the lamina propria of the gastrointestinal tract, where they appear as plasma cells.

After further investigation, Isaacson and Wright noticed that the cells previously thought to be follicle centre cells, in fact were not, because they did not contain cytoplasmic immunoglobulin, despite their common surface markers. Therefore,

these cells were designated "centrocyte-like cells" (CCL cells)⁴⁰. These cells invaded individual glands to form the characteristic lymphoepithelial lesions, studied in detail by Papadaki⁵⁴, and could also selectively colonise reactive follicles in MALT lymphoma, rendering these follicles a monotypic (i.e. showing light chain restriction) appearance and hence a striking resemblance to follicular lymphoma⁵⁵⁻⁵⁷.

Furthermore, they advocated to abandon the term pseudolymphoma, because the term is vague and subjective and it should be possible in almost any case to determine whether lymphoma was present using new techniques⁵⁸. Besides, they reasoned: "the relatively 'benign' behaviour of former gastric pseudolymphoma is to be expected in view of their derivation from the mucosa-associated lymphoid tissue and is certainly no reason for these lesions to be really benign".

In 1987 and 1990 they reviewed the morphological features of mucosa-associated tissue and reported a striking resemblance of the centrocytelike cell with splenic marginal zone cells. The latter surrounded B-cell follicles and also resembled centrocytes cytologically^{59,60}. These centrocyte-like cells in the normal gut-associated lymphoid tissue form focal intraepithelial collections in dome epithelium, which could represent a benign form of lymphoepithelial lesion. The marginal zone as possible cell of origin could also explain the long localized period of primary gastric lymphomas, because in rodents, it has been shown that marginal zone cells do not recirculate⁶¹.

Intermezzo I - The normal mucosa-associated lymphoid tissue

Formations of lymphocytes are found throughout the whole length of the human gut: they include the aggregated lymphoid tissues such as tonsils, Peyer's patches and appendix, diffuse collections of plasma cells and lymphocytes within the lamina propria, and the separate lymphocytes between epithelial cells covering the villi (the intra-epithelial lymphocytes)^{12,62,63}.

Peyer's patches were first described by Peyer in 1667, and in the human intestines about 200 are found, most concentrated in the terminal ileum⁶⁴. Peyer's patches extend through the lamina propria and submucosa of the small intestine, and crypts and villi are sparse in the overlying mucosa. The part of the epithelium which is immediately above the nodules in Peyer's patches is flattened and heavily infiltrated with lymphocytes and is known as the dome area. In the dome epithelium, epithelial cells characterized by luminal surface microfolds (M cells) are found rather than epithelial cells with microvilli, and these are thought to be important for transport of antigenic material and traffic of lymphocytes⁶⁵.

Whereas B-cell follicles in normal human lymphoid tissue consist of germinal centre and mantle zone lymphocytes (the presence of a marginal zone in human reactive lymph nodes is controversial⁶⁶), the human gut-associated lymphoid tissue contains extrafollicular B-cells which are distinct from the mantle zone lymphocytes because they morphologically resemble germinal centre cells (centrocytes), they lack surface IgD and

they invade the epithelium over the dome regions of the lymphoid follicles⁶⁷. Because cells with these characteristics were also observed in primary B-cell lymphomas of the mucosa⁵³, Spencer and colleagues compared them. They found that these cells surrounded the follicles in Peyer's patches, merging with the mantle zone and extending towards the dome region as well as towards the serosa. They concluded that these cells which resembled centrocytes were identical to the B-cells in the marginal zone of the spleen⁶⁸, and are therefore believed to be the mucosal counterparts of marginal zone cells in the spleen. The function of these marginal zone cells is not yet elucidated, but in rodents they have been found to be non-circulating lymphocytes⁶¹. They could be precursors of antibody-forming cells and play a role in the transport of antigen-antibody complexes⁶⁹.

A further point of interest was the relation between low and high grade primary gastrointestinal lymphomas, as it was noticed that the majority of these lymphomas were of high grade malignancy.

The question was addressed whether the large cell lymphomas of B-cell lineage which account for the majority of gastric lymphomas originate from small cell MALT lymphomas, or arise de novo in the stomach. Chan conducted a study of 10 cases of primary malignant lymphoma of the stomach in which a small cell as well as a large cell component were present and found that in all of these cases both components showed identical light chain restriction. He concluded from these results that the high grade component develops through blastic transformation of the low-grade component⁷⁰. Other studies supporting a clonal link between low grade and high grade gastric lymphoma came from Montalbán et al.⁷¹ showing a clonal IgH rearrangement of the same size using the polymerase chain reaction (PCR) in a low grade case progressing into high grade lymphoma and from Peng et al.⁷² which also used PCR to identify clone-specific rearranged IgH gene sequences in four lymphomas with both low and high grade components.

A problem exists in those high grade cases in which no associated low grade component can be detected, as in these cases it is impossible to distinguish high grade lymphomas of MALT from nodal centroblastic or immunoblastic lymphoma, at least on histological grounds⁶⁰. There can be various reasons for not detecting a low grade component: the high grade lymphoma may have overgrown the low grade lymphoma, not all significant parts of the lymphoma have been enclosed in paraffin, or a de novo high grade lymphoma could have developed⁷⁰.

With the growing understanding of primary gastrointestinal lymphomas and the use of the MALT concept, classifications were adjusted and designed to incorporate these lymphomas.

New classification systems were designed, incorporating the concept of lymphomas arising from the mucosa-associated lymphoid tissue. The first approach was to modify the Kiel-classification by adding two categories: Mal(Low) and Mal(High) lymphoma. Several studies followed to assess the importance of these modifications

with various conclusions⁷³.

In 1993, on the 5th International Conference on Malignant Lymphoma a consensus meeting was organized to discuss the pathological and staging classifications of gastrointestinal tract lymphoma⁷⁴. It was recommended that the histological classification described by Isaacson⁷⁵ be adopted, because of its relatively simple use and because it follows the principles of the KIEL classification (Table 1.1).

Table 1.1 Classification of primary gastrointestinal lymphoma.

Classification of primary gastrointestinal lymphoma (Isaacson, 1988 ⁷⁵)	
<hr/>	
<i>B cell</i>	Low grade B cell lymphoma of MALT High grade B cell lymphoma of MALT, with or without evidence of a low grade component Immunoproliferative small intestinal disease (IPSID), low grade, mixed or high grade Mantle Cell lymphoma Burkitt like lymphoma Other types of low or high grade lymphoma corresponding to peripheral lymph node equivalents
<i>T cell</i>	Enteropathy associated T cell lymphoma (EATCL) Other types not associated with lymphadenopathy

One year later, a proposal from the International Lymphoma Study Group (REAL Classification) was published based on the combination of current understanding of histology, immunology, genetics, clinical presentation, course and putative normal counterpart in the immune system. On the basis of these findings, entities were formulated and low grade mucosa-associated lymphoid tissue lymphomas were classified as marginal zone B-cell lymphomas¹⁰, and believed to originate from memory B-cells residing in the marginal zones of mucosal lymphoid tissues. In this classification system, high grade MALT lymphomas were not mentioned and most large cell lymphomas occurring in the gastrointestinal tract must be classified as diffuse large B-cell lymphomas.

Acquired MALT and *Helicobacter pylori*

As mentioned earlier, the normal stomach is devoid of any organized lymphoid tissue, whereas the majority of primary gastrointestinal lymphomas originates in the stomach. On the other hand, only few lymphomas arise primarily in the terminal ileum which has the highest concentration of gut associated lymphoid tissue⁷⁶. For a long time, no attention was paid to this paradoxical distribution of primary gastrointestinal lymphoma, but recently, more and more investigators have been addressing the acquisition of mucosa-associated lymphoid tissue in the stomach.

At the time Isaacson and colleagues defined lymphomas of mucosa-associated lymphoid tissue⁵³, Warren⁷⁷ and Marshall⁷⁸ were the first to culture 'unidentified curved bacilli' present on gastric epithelium in chronic active gastritis. They noticed a resemblance with *Campylobacter jejuni*. Because of this resemblance the

bacterium was first named *Campylobacter pylori* to correspond to the nomenclature of other enteric pathogens. In 1989, it was reclassified and renamed as *Helicobacter pylori*, because of distinct functional and enzymatic properties.

Research on *H.pylori* has expanded exponentially, and at this moment, there is general agreement that the organism plays a causative role in the pathogenesis of gastritis, duodenal ulcer and gastric cancer⁷⁹⁻⁸². In an epidemiological study, an unexpected link with primary gastric lymphoma was noticed, which instigated research on the role of *H.pylori* in primary gastric lymphoma⁸³.

Intermezzo II - *Helicobacter pylori*

Bacteriology

H.pylori is a Gram-negative, microaerophilic, curved, S-shaped or spiral bacillus with 1-5 sheathed unipolar flagella. The bacteria are about 0.5-1.0 μm in length. A very characteristic feature of *H.pylori* is a very strong urease activity which creates a local alkaline microenvironment. *H.pylori* uses its flagella to drill through the mucus layer and to migrate towards regions of moderate acidity. Bacterial heterogeneity is considerable, raising the possibility that different strains produce different clinical conditions⁸⁴⁻⁸⁶.

Transmission and epidemiology

H.pylori is mainly transmitted by the orofecal route. In developing countries water seems an important vector and the prevalence is still rising to approximately 75% by the mid-teens remaining constant thereafter. In Western countries, the prevalence of *H.pylori* infection gradually increases with age, from 10% in young adults to a peak of 70% in the eighth decade of life^{85,87-89}.

Mechanisms by which *H.pylori* can damage the gastric mucosa

In *H.pylori* gastritis, a direct correlation exists between numbers of bacteria on the mucosa and the extent of the inflammatory response in the infected area. Probably, bacterial products are directly responsible for recruitment of inflammatory cells into the gastric mucosa. The way in which *H.pylori* damages the gastric mucosa is not yet fully elucidated. Possible mechanisms are the formation of adhesion sites between *H.pylori* and epithelial cells, cytotoxin production by some bacterial strains, chemical injury from ammonia (produced by urease activity), mucolysis, damage by free oxygen metabolites, or other pathogenetic mediators^{79,85,90-93}.

Diagnosis of *H.pylori*

Several tests are available for the detection of *H.pylori*. The biopsy urease test and the urease breath test use the characteristic urease production of the bacteria and respectively detect this enzyme in biopsies and in exhaled CO_2 . Culture is another possibility, which allows antibiotic sensitivity to be determined. Furthermore, antibodies against *H.pylori* can be detected in serum or saliva using ELISA kits. A very sensitive way to detect *H.pylori* is the polymerase chain reaction (PCR). A diagnostic procedure which deserves

special attention is histology. In histological sections, *H. pylori* is diagnosed by its uniform, curved, U-shaped or seagull shaped appearance in close relation with the surface epithelium. *H. pylori* can be detected in routine haematoxylin-eosin stains, but is more easily seen in Warthin-Starry, Giemsa, Steiner, Cresyl fast violet, Brown-Hopps and 'Half-Gram' stains. In principle only '*H. pylori*-like' organisms can be detected by these non-specific staining methods and only specific staining techniques like immunoperoxidase and in situ hybridisation techniques can unequivocally establish the exact nature of the organism. Especially cases with very low numbers of *H. pylori* or patchy distribution of the bacteria are difficult to diagnose^{85,90,94}.

Parsonnet and colleagues therefore conducted a similar study with special reference to the role of *H. pylori* in primary gastric lymphoma and found that patients with gastric non-Hodgkin's lymphoma were substantially more likely than matched controls to have evidence of prior infection with *H. pylori*⁹⁵. Other evidence for a possible role of *H. pylori* in low grade primary gastric lymphoma has come from three directions: first, the increased presence of lymphoid follicles and aggregates in *H. pylori* infected stomachs (which could be a form of acquired functional mucosa associated lymphoid tissue)⁹⁶⁻⁹⁸, second, the histological presence of *H. pylori* in gastric mucosa adjacent to primary gastric lymphoma^{99,100} and third, regression of primary gastric low grade MALT lymphoma after eradication therapy for *H. pylori*¹⁰¹⁻¹⁰³. However, not all studies point in the same direction, for example in studies concerning the eradication therapy, the follow-up periods are still very short and recurrences of lymphoma after eradication of *H. pylori* are also reported¹⁰⁴. Furthermore, the usefulness of eradication therapy in high grade primary gastric lymphoma has to be assessed as well¹⁰⁵. Finally, about the occurrence of *H. pylori* in patients with gastric lymphoma (the second line of evidence) some disagreement remains as well. Some studies report the presence of *H. pylori* in over 90% of the cases, even up to 100% and on the other hand percentages of 41% and 50% are reported^{106,107}.

In conclusion, although there are data supporting the role of *H. pylori* in primary gastric lymphoma suggesting an infectious nature or origin of primary gastric lymphoma^{108,109}, there are still some controversies. One of these is that although a majority of the population carries *H. pylori*, only a minority develops primary gastric lymphoma, making other contributing factors in tumorigenesis plausible.

Epidemiology

The incidence and the mortality from non-Hodgkin's lymphoma in general is rising^{110,111}. It has been suggested that this increase is mainly due from a rising incidence of primary extranodal and in particular of gastric lymphoma¹¹²⁻¹¹⁵. The reported incidence of primary extranodal non-Hodgkin's lymphoma in Western countries ranges from 15% up until 84% of all non-Hodgkin's lymphomas¹¹⁶⁻¹¹⁸. The

majority of these extranodal lymphomas arise in the tonsils (when considered extranodally), followed by the gastrointestinal tract, skin and bone¹¹⁷. Other sites of involvement are the central nervous system, liver, thyroid, lungs, salivary glands, testes, connective tissue, nasal cavity, uterus, breast, orbit, pancreas and oral cavity¹¹⁹. Although primary gastrointestinal non-Hodgkin's lymphoma accounts for a majority of primary extranodal lymphoma it accounts for only 4-18%¹²⁰⁻¹²² of all non-Hodgkin's lymphomas. Primary gastric lymphoma accounts for between 1-5% of all gastric malignancies¹²³.

Moreover, there is a marked geographical variation in the incidence of primary extranodal¹²⁴ as well as primary gastrointestinal lymphoma, the latter showing a high incidence in the Middle East¹²⁵. In Western Europe some areas like the north-east of Italy show a high incidence as well¹²⁶.

Primary gastric lymphoma accounts for the majority (two-third) of primary gastrointestinal lymphoma, primary intestinal lymphoma for the remaining one-third. In intestinal lymphoma, the small intestines are involved twice as often as the colon^{127,128}. Primary small intestinal lymphomas are rather common among all small intestinal malignancies, being the third most common after adenocarcinomas and carcinoid tumours¹²⁹. Colorectal lymphoma accounts for a small minority of colon neoplasms, only 11% out of the three percent of colon neoplasms other than adenocarcinomas^{130,131}.

The incidence of primary intestinal lymphoma is also rising, in particular among immunocompromised patients, like those suffering from AIDS, or those who have received immunosuppressive therapy, for example transplant recipients^{132,133}.

Primary gastrointestinal lymphoma as a clinical entity

Comparison of clinical studies on primary gastrointestinal lymphoma is necessary to give an adequate description of the clinical features of gastric and intestinal lymphoma. Unfortunately, the different systems used for the pathological classification of primary gastrointestinal lymphoma renders this very difficult. Moreover, these difficulties are not limited to pathological classification systems, but are also encountered in the staging classifications. Before presenting the clinical features, an overview of the developments and ideas in staging classifications will be given.

Staging in Hodgkin's Disease, non-Hodgkin's lymphoma, primary extranodal and primary gastrointestinal lymphoma.

Staging in neoplastic diseases is important for several reasons, such as providing important prognostic information, assisting the physician in selecting the most appropriate therapeutic strategies and the availability of a standardized stem which permits comparisons of therapeutic results¹³⁴. One of the first staging systems in Hodgkin's disease was the Rye classification, adopted at a conference in Rye, New

York in 1965 (Symposium Obstacles to the Control of Hodgkin's Disease: Rye, New York, September 13-15, 1966)¹³⁵, which was revised and completed in 1971 at the Ann Arbor symposium to make the classification applicable to all malignant lymphomas (Symposium "Staging in Hodgkin's Disease": Ann Arbor, Michigan, April 26-28, 1971)¹³⁶.

Table 1.2 Modification of the Ann Arbor classification by Peters et al.¹³⁷

Lymph node pattern	Stage	Extranodal pattern
Single region	I	Single extranodal site
More than one region in upper or lower torso	II	Single site + regional nodes
Regions in upper and lower torso	III	One or two sites and lymph nodes beyond regional
Advanced disease with metastases (liver, lung, bone marrow, etc.)	IV	Advanced disease with metastases

I-IV A: without systemic effects; I-IV B: with systemic effects

Musshoff in 1971, and Peters in 1974 recognized that, although nodal and extranodal lymphomas in general share the same pathogenesis, a few fundamental differences exist, like a favourable prognosis of extranodal lymphomas in the local and regional stages¹¹⁷, and therefore proposed modification of the Rye classification and the Ann Arbor classification^{137,138}. Peters and his colleagues proposed a division of stages I-III of the Ann Arbor classification into nodal and extranodal disease, because of the different pattern of spread in both modalities. Further, they suggested limitation of stage II extranodal disease to the primary site with involvement of the regional lymph nodes only (Table 1.2). This modification was suggested because of the difference to nodal disease with regional lymph nodes only, in which cures or long time survivors, at that time, were not observed, whereas in extranodal stage II they were.

In 1975 Musshoff and Schmidt-Vollmer evaluated whether and to what extent the Ann Arbor classification was indeed applicable to all malignant lymphomas¹³⁹. They found that in essence the Ann Arbor classification reflects the oncological properties of non-Hodgkin's lymphomas, but reported two major problems: firstly, that the dissemination stages in non-Hodgkin's lymphoma are more dependent on histological type than in Hodgkin's disease and secondly the extranodal origin of a significant part of the non-Hodgkin's lymphomas. Concerning the latter problem, they also reflected on Peters's modifications of the Ann Arbor Classification. They agreed on the major division in nodal and extranodal disease, but did not agree on the limitation of stage II extranodal disease to the primary site including regional

lymph nodes. However, they thought it worth considering to divide stage II into stage II₁ and stage II₂, the latter with involvement of more than two contiguous anatomical regions or of two or more non-contiguous regions on the same side of the diaphragm. In summary, they suggested the staging classification shown in table 1.3. In 1977 this classification was again renewed¹⁴⁰. The most important modification was the division of stage I into stage II (tumour infiltration limited to mucosa and/or submucosa) and I2 (tumour infiltration into muscularis propria and/or subserosa and/or serosa).

Table 1.3 Modified Ann Arbor classification.

Lymph node pattern	Stage	Extranodal pattern
Single lymph node region	I	Single extralymphatic organ and site
Two contiguous lymph node regions on the same side of the diaphragm (II ¹) or one lymph node region with localized involvement of one extralymphatic organ and site (II ² e)	II ¹	Single extralymphatic organ with regional lymph node (II ¹) or contiguous extralymphatic site (II ¹ e)
More than two contiguous lymph node regions or two non-contiguous lymph node regions on the same side of the diaphragm (II ²), including localized involvement of extralymphatic organ and site (II ² e)	II ²	Extralymphatic organ and lymph node involvement, extending beyond regional lymph node involvement on the same side of the diaphragm (II ²), including localized extralymphatic site (II ² e)
Involvement of lymph node regions on both sides of the diaphragm (III) which may also be accompanied by localized involvement of extralymphatic organ or site (IIIe) or by involvement of the spleen (IIIs) or both (IIIse)	III	Involvement of extralymphatic organ and site and lymph node regions on both sides of the diaphragm, which may also be accompanied by (second) localized involvement of extralymphatic organ or site (IIIe) or by involvement of the spleen (IIIs) or both (IIIse)
Lymph node involvement with diffuse or disseminated involvement of extralymphatic organs and tissues	IV	Diffuse or disseminated involvement of 1 or more extralymphatic organs and tissues with or without associated lymph node involvement.

I-IV A: without general symptoms, I-IV B: with general symptoms according to the Ann Arbor definition

In 1979 Blackledge and colleagues set up a separate staging system for primary gastrointestinal lymphoma¹⁴¹, because in their opinion, conventional staging systems like the Ann Arbor system are only of limited value in these lymphomas whereas the prognosis of the patient depends mainly on the extent of disease within the abdomen. They proposed the staging system described in table 1.4.

Table 1.4 Staging of gastrointestinal lymphoma (Blackledge et al.¹⁴¹).

Stage	Description
IA	Tumour confined to gastrointestinal tract
IB	Multiple tumours confined to gastrointestinal tract
IIA	Tumour with local nodes (gastric or mesenteric)
IIB	Tumour with perforation and adherence to adjacent structures
IIC	Tumour with perforation and peritonitis
III	Tumour with widespread nodal involvement (para-aortic or more distant nodes)
IV	Tumour with disseminated disease (e.g. liver, bone marrow involvement)

As can be noticed from the above, comparison of results from studies on gastrointestinal lymphoma remains difficult because of the lack of consensus on classification and staging. In 1993 on the Vth International Conference on Malignant Lymphoma, this problem was discussed, together with the problems on pathological classifications as described earlier in this chapter⁷⁴.

The staging classification as outlined in table 1.5 was recommended. Stage III disease was deleted after discussing its relevance in the context of primary gastrointestinal lymphoma. Furthermore, the subscript E was used in its original meaning, denoting proximal or contiguous extranodal disease that could be included within an irradiation field appropriate for nodal disease of the same anatomical extent.

After the publication of this staging classification another staging classification based upon the TNM classification has been suggested by Shimodaira and colleagues¹⁴² as follows: T1, tumour invades the lamina propria or submucosa; T2, the muscularis propria; T3, the subserosa; T4, the serosa without invasion of adjacent structures; T5, adjacent structures. N0, no regional lymph node metastasis; N1, perigastric lymph nodes within 3 cm of the edge of the tumour; N2, perigastric lymph nodes more than 3 cm from the edge of the primary tumour or in lymph nodes along the left gastric, common hepatic, splenic or celiac arteries; N3, paraaortic and hepatoduodenal lymph nodes and/or other intraabdominal lymph nodes; N4, beyond N3. M0, no distant metastasis; and M1, positive. They found this staging system useful in assessing prognosis and deciding about a therapeutical approach in primary gastric lymphoma.

As can be concluded from these varying classification systems, there is still little agreement on staging in primary gastrointestinal lymphoma. This and the discussion on the pathological staging systems make comparisons between studies very difficult and therefore, the clinical picture of primary gastrointestinal lymphoma will be discussed only briefly.

Table 1.5 Staging classification for primary gastrointestinal lymphoma after a consensus meeting in Lugano 1993⁷⁴.

Stage	Description
I	Tumour confined to the GI tract: single primary site, or multiple non-contiguous lesions.
II	Tumour extending into abdomen from primary GI site: II ₁ : Local nodal involvement: paragastric in cases of gastric lymphoma and para-intestinal for intestinal lymphoma II ₂ : Distant (mesenteric in the case of an intestinal primary, otherwise: para-aortic, para-caval, pelvic, inguinal)
III	Penetration of serosa to involve adjacent organs or tissues (enumerate actual site of involvement, e.g. IIIE _(pancreas)) and in cases of both nodal involvement and penetration to involve adjacent organs, stage should be noted using both a subscript (1 or 2) and E)
IV	Disseminated extranodal involvement, or, a GI tract lesion with supra-diaphragmatic nodal involvement.

Despite the fact that a lot of studies described clinical features in gastrointestinal lymphoma as a group^{123,128,143-146}, it has become evident that gastric and intestinal lymphomas behave differently to such an extent that it is more efficient to discuss them separately.

Clinical features of primary gastric NHL

Usually, patients present with non-specific symptoms, like pain, nausea, vomiting, dyspepsia and anorexia¹⁴⁷⁻¹⁵⁰. These symptoms can also be found in peptic ulcer disease and gastric carcinoma. Haemorrhage is less frequent, but may be seen in up till 38% of the patients¹⁵¹. Perforation as the first presentation is unusual, generally found in less than 5% of the patients¹⁵². The endoscopic pattern in gastric lymphoma is not specific for lymphoma. For example in the series of Taal et al.¹⁵³, low grade gastric lymphoma often resembled peptic ulcer disease or gastritis. In the same study, it was found that in high grade gastric lymphoma, malignancy was more often suspected.

Treatment of primary gastric lymphoma remains controversial, and the effectiveness of different modes of treatment needs further assessment. Many studies focus on one treatment modality (surgery, chemotherapy or radiotherapy). In high grade gastric lymphoma, there are contradictory results, in favour of gastrectomy alone^{127,147,154,155}, combination of gastrectomy and chemotherapy^{156,157} and in favour of chemotherapy or radiotherapy without surgery¹⁵⁸⁻¹⁶⁰. The fact that a number of tumours is not resectable at surgery^{127,154} and that complications and morbidity as a result of gastrectomy are relatively frequent^{156,159} makes surgery less

attractive. Of course the role of *Helicobacter pylori* eradication therapy is currently under discussion. Regression of primary low-grade gastric lymphoma of MALT has been shown after eradication therapy for *Helicobacter pylori*^{101-103,161}. Furthermore, regression of low grade MALT lymphomas arising at other extranodal sites like the salivary gland, the rectum, the duodenum and regression of IPSID has also been described following *Helicobacter pylori* eradication therapy¹⁶²⁻¹⁶⁵. However, long-term follow-up is still unknown and the role of this therapy in small and large cell primary gastric lymphoma has to be further investigated.

Clinical features of intestinal lymphoma

Primary intestinal non-Hodgkin's lymphoma is a rare disease. Moreover, unlike gastric lymphoma it is a very heterogenous group of lymphomas, leading to even more uncommon sub-entities. This is due to their localisation in the intestinal tract, i.e. small intestinal NHL¹⁶⁶⁻¹⁶⁸ and colon and rectum NHL^{131,169-171}. Histologically they present as MALT lymphoma, mucosal mantle cell lymphoma^{172,173}, enteropathy-associated T-cell lymphoma¹⁷⁴⁻¹⁷⁶, or as immunoproliferative small intestinal disease (IPSID)^{177,178}.

Like gastric lymphoma, intestinal lymphoma often presents with non-specific abdominal pain and weight loss. Signs of disseminated lymphoma like lymphadenopathy and hepatosplenomegaly are usually absent. However, perforation is much more common in primary intestinal lymphoma, and may occur in up to one quarter of the cases. Obstruction may also occur, and is more common in small bowel than in large intestinal lymphoma^{179,180}. Because of this acute presentation intestinal lymphoma often requires surgical therapy, but it has been shown that this rarely eradicates the disease, because the disease is often advanced. Therefore, adjuvant therapy may often be necessary. Chemotherapy can give a better long-term survival and less morbidity compared to radiation therapy¹⁷⁹. Finally, like in gastric lymphoma, the role of *Helicobacter pylori* eradication therapy has to be further investigated.

Questions addressed in this study

From the preceding review it appears that although questions about definition, classification, staging and clinical behaviour have been addressed for a long time by several investigators, there still remain several issues to be resolved regarding gastrointestinal lymphoma. Because of the ongoing confusion and discussion between pathologists and clinicians about these questions our goal was to investigate pathological and clinical features of these lymphomas to try and shed more light on their definition, classification, clinical behaviour and pathogenesis.

First we studied whether the distinction between primary gastrointestinal lymphomas of MALT origin and primary gastrointestinal lymphoma without recognisable features of MALT is useful in regard of classification, diagnosis and prediction of clinical outcome (*Definition*).

Second, we sought for other than histopathological clues to make a distinction between lymphomas with and without recognisable features of MALT, considering that biopsies taken in endoscopic procedures often do not contain enough material to make a proper distinction between MALT and non-MALT (*Classification*).

Third, we analyzed if from these findings one is able to elucidate if and why MALT lymphomas differ clinically and pathologically from non-MALT lymphomas (*Clinical behaviour*).

Furthermore, given the assumed antigen driven origin of MALT lymphomas we investigated which organisms apart from *Helicobacter pylori* could be responsible for and are still present in the lymphoma stage (*Pathogenesis*).

An additional point of interest was whether the difference in localisation of the lymphoma is important for clinical behaviour and also accounts for differences in pathogenesis between stomach and intestinal lymphoma, based on the clinical and pathological data gathered in our series.

Outline of this study

Proliferation and apoptosis in primary gastric lymphoma (Chapter 3)

In chapter 3 we sought to elucidate the role of cell proliferation and apoptosis in the pathogenesis of gastric lymphoma (*Classification*). Cell proliferation rate is an important prognostic factor in many neoplastic diseases, and non-Hodgkin's lymphoma is no exception¹⁸¹⁻¹⁸⁴. Another factor determining the growth of a tumour is cell death, and in particular programmed cell death or apoptosis. In nodal non-Hodgkin's lymphoma it has been found that proliferation and apoptosis positively correlate with each other¹⁸⁵. We therefore studied the role of proliferation, apoptosis as well as their interrelation in primary gastric lymphoma.

Microbiological studies in primary gastric lymphoma (chapter 4 & 5)

As previously described in this chapter, the stomach is normally devoid of any organized lymphoid tissue, but still, most gastrointestinal lymphomas arise in the stomach. It has been hypothesized that a chronic active *Helicobacter pylori* gastritis can lead to the formation of 'acquired' mucosa-associated lymphoid tissue in the stomach^{96,108,186,187}. Although there are many data supporting this hypothesis, the occurrence of *H.pylori* has only been extensively studied in low-grade gastric MALT lymphoma, whereas the majority of gastric lymphomas is of high grade histology. We therefore assessed whether *H.pylori* could also be shown in large cell primary gastric lymphomas (*Pathogenesis*). Furthermore, all previous studies

on the histological presence of *H. pylori* in the stomachs of patients with gastric lymphoma have been performed using non-specific staining methods for *H. pylori*, making the evaluation unreliable or difficult to interpret. We used a specific as well as sensitive immunohistochemical staining to detect *H. pylori*.

Besides *H. pylori*, the presence of other micro-organisms could be assessed as well, by joint evaluation of both the specific immunological staining as well as an non-specific modified Giemsa staining.

Molecular biological studies in primary gastric lymphoma (Chapter 6)

To address the question whether there are other than histological features to distinguish MALT from non-MALT lymphoma, in particular in the high grade cases, in which the diagnosis MALT often depends on the availability of tissue, our objective is to study numerical chromosomal abnormalities in MALT lymphoma. This is based on earlier studies, reporting about a recurring trisomy of chromosome 3 in low grade MALT lymphoma, and less often trisomies of chromosome 12 and 18¹⁸⁸. To confirm these results and to be able to compare small and large cell primary gastric lymphomas and large cell lymphomas with or without a small cell MALT component, we used fluorescent (FISH) and brightfield *in situ* hybridisation (ISH) to detect numerical chromosomal abnormalities (*Classification*).

Clinicopathological correlations in primary gastric lymphoma (Chapter 7)

In this chapter, the clinical features of this patient group are described and furthermore, data gathered in the previous chapters are discussed in relation to the clinical picture to try and explain the different clinical behaviour of gastric lymphomas (*Clinical behaviour*).

Cell turnover studies in primary intestinal lymphoma (Chapter 8)

Proliferation and apoptosis were also studied in primary intestinal lymphoma, in which special attention was given to differences between localisation, MALT and non-MALT cases and different entities in this very heterogeneous group of neoplasms (*Classification, definition*).

We used the same techniques as in chapter 3, in order to be able to compare these findings to the data on gastric lymphoma.

Clinicopathological studies in primary intestinal lymphoma (Chapter 9)

In this chapter we describe the clinical picture of intestinal lymphoma and we again correlate these data to the data on cell turnover of chapter 8. In addition, prognostic clinical factors are studied (*Clinical behaviour*).

References

1. Rappaport H. Tumors of the hematopoietic system. Atlas of tumor pathology. Washington: Armed Forces Institute of Pathology, 1966.
2. Bennett MH, Farrer-Brown G, Henry K, Jelliffe AM. Classification of non-Hodgkin's lymphomas. *Lancet* 1974;405-406.
3. Dorfman RF. Classification of non-Hodgkin's lymphomas. *Lancet* 1974;1295-1296.
4. Gérard-Marchant R, Hamlin I, Lennert K, Rilke F, Stansfeld AG, VamUnnik JAM. Classification of non-Hodgkin's lymphomas. *Lancet* 1974;406-408.
5. Lukes RJ, Collins RD. A functional approach to the classification of malignant lymphoms. *Recent Res Cancer Res* 1974;46:18-30.
6. Mathé G, Rappaport H, O'Connor GT, Torloni H. Histological and cytological typing of neoplastic diseases of haematopoietic and lymphoid tissues. *International Histological Classification of Tumors* 1976;14.
7. Lennert K, Mohri N, Stein H, Kaiserling E, Müller-Hermelink HK. Malignant lymphomas other than Hodgkin's disease. Berlin, Heidelberg, New York: Springer Verlag, 1978.
8. Rosenberg SA. National Cancer Institute sponsored study of classifications of non-Hodgkin's lymphomas. Summary and description of a Working Formulation for clinical usage. *Cancer* 1982;49:2112-2135.
9. Stansfeld AG, Diebold J, Kapanci Y, Keleny G, Lennert K, Mioduszezowska O, Noel H, Rilke F, Sundstrom C, Van Unnik JAM, Wright DM. Updated Kiel classification for lymphomas. *Lancet* 1988;6:292-293.
10. Harris NL, Jaffe ES, Stein H, Banks PM, Chan JKC, Cleary ML, Delsol G, De Wolf-Peeters C, Falini B, Gatter KC, Grogan TM, Isaacson PG, Knowles DM, Mason DY, Muller-Hermelink HK, Pileri SA, Piris MA, Ralfkiaer E, Warnke, RA. A revised European-American Classification of Lymphoid Neoplasms: A proposal from the international lymphoma study group. *Blood* 1994;84:1361-1392.
11. Chan JKC, Banks PM, Cleary ML, Delsol G, De Wolf-Peeters C, Falini B, Gatter KC, Grogan TM, Harris NL, Isaacson PG, Jaffe ES, Knowles DM, Mason DY, Muller-Hermelink HK, Pileri SA, Piris MA, Ralfkiaer E, Stein H, Warnke RA. A proposal for classification of lymphoid neoplasms (by the International Lymphoma Study Group). *Histopathology* 1994;1994:517-536.
12. Parrott DMV. The Gut as a lymphoid organ. *Clin Gastroenterol* 1976;5:211-228.
13. Virchow R. Die Krankhaften Geschwulste. Berlin: Hirschwald, 1863;2:728-738.
14. Hulbert. Specimen of sarcoma of the viscera. *St. Louis Med Surg J* 1885;xlvi:250.
15. Crowther C. Studio dei sarcome primitivo dell intestino tenue con contributio di tre casi originale. *Clin Chir Milano* 1913;21:2107.
16. Graves S. Primary lymphoblastoma of the intestines. Report of three cases, one with apparent recovery following operation. *J Med Res* 1919;xl:415.
17. Ullman A, Abeshouse BS. Lymphosarcoma of the small and large intestines. *Ann Surg* 1932;95:878-915.
18. Ewing J. Neoplastic Diseases. (4 ed.) Philadelphia: W.B. Saunders Company, 1940.

19. Madding GF, Walters W. Lymphosarcoma of the stomach. *Arch Surg* 1940;40:120-134.
20. Sugarbaker ED, Craver LF. Lymphosarcoma: a study of 196 cases with biopsy. *JAMA* 1940;115:17-23.
21. Smith JL, Helwig EB. Malignant lymphoma of the stomach; its diagnosis, distinction and biologic behaviour. *Am J Pathol* 1958;34:553.
22. Jacobs DS. Primary gastric malignant lymphoma and pseudolymphoma. *Am J Clin Pathol* 1963;40:379-394.
23. Faris TD, Saltzstein SL. Gastric lymphoid hyperplasia: a lesion confused with lymphosarcoma. *Cancer* 1964;17:207-212.
24. Johnson AG, Sowerbutts JG. Pseudolymphoma of the stomach. *Brit J Surg* 1968;55:81-83.
25. Azzopardi JG, Menzies T. Primary malignant lymphoma of the alimentary tract. *Brit J Surg* 1960;47:358-366.
26. Berry GR, Mathews WH. Gastric lymphosarcoma and pseudolymphoma: Reappraisal of 12 cases of gastric lymphosarcoma. *Can Med Assoc J* 1967;96:1312-1316.
27. Perez CA, Dorfman RF. Benign lymphoid hyperplasia of the stomach and duodenum. *Radiology* 1966;87:505-510.
28. Kay S. Lymphoid tumors of the stomach. *Surg Gynecol Obstet* 1964;118:1059-1066.
29. Anderson JR, Lee D, Naysmith A, Busuttil A. Gastric pseudolymphoma. *Brit J Surg* 1980;67:672-674.
30. Highman LM, Fantelli FJ, Hermann RE. Pseudolymphoma of the stomach. *Arch Surg* 1981;116:227-229.
31. Ranchod M, Lewin KJ, Dorfman RF. Lymphoid hyperplasia of the gastrointestinal tract. A study of 26 cases and review of the literature. *Am J Surg Pathol* 1978;2:383-400.
32. Valdés-Dapena A, Affolter H, Vilardell F. The gradient of malignancy in lymphoid lesions of the stomach. *Gastroenterology* 1966;50:382-389.
33. Kahn LB, Novis BH. Nodular lymphoid hyperplasia of the small bowel associated with primary small bowel reticulum cell lymphoma. *Cancer* 1974;33:837-844.
34. Wolf JA, Spjut HJ. Focal lymphoid hyperplasia of the stomach preceding gastric lymphoma: Case report and review of the literature. *Cancer* 1981;48:2518-2523.
35. Hurst DW, Meyer OO. Giant follicular lymphoblastoma. *Cancer* 1961;14:753-778.
36. Akagi T, Okajima K, Fujii Y, Ishiwaka M. Giant follicular lymphoma of the stomach. Report of four cases. *Acta Pathol Jpn* 1973;23:397-405.
37. Causey JQ. The benign lymphoepithelial lesion - A harbinger of neoplasia. *Southern Med J* 1976;69:60-63.
38. Salmon SE, Seligmann M. B-cell neoplasia in man. *Lancet* 1974;1230-1233.
39. Burke JS, Sheibani K, Nathwani BN, Winberg CD, Rappaport H. Monoclonal small (well differentiated) lymphocytic proliferations of the gastrointestinal tract resembling lymphoid hyperplasia. A neoplasm of uncertain malignant potential. *Hum Pathol* 1987;18:1238-1245.

40. Isaacson PG, Spencer J, Finn T. Primary B-cell gastric lymphoma. *Hum Pathol* 1986;17:72-82.
41. Sigal SH, Saul SH, Auerbach HE, Raffensperger E, Kant JA, Brooks JJ. Gastric small lymphocytic proliferation with immunoglobulin gene rearrangement in pseudolymphoma versus lymphoma. *Gastroenterology* 1989;97:195-201.
42. Dawson IMP, Cornes JS, Morson BC. Primary malignant lymphoid tumours of the intestinal tract. Report of 37 cases with a study of factors influencing prognosis. *Brit J Surg* 1961;49:80-89.
43. Henry K, Farrer-Brown G. Primary lymphomas of the gastrointestinal tract. I. Plasma cell tumours. *Histopathology* 1977;1:53-76.
44. Yamanaka N, Ishii Y, Koshiba H, Suzuki T, Ogasawara M, Arisul T, Kikuchi K. A study of surface markers in gastrointestinal lymphoma. *Gastroenterology* 1980;79:673-677.
45. Grody WW, Weiss LM, Warnke RA, Magidson JG, Hu E, Lewin KJ. Gastrointestinal lymphomas. Immunohistochemical studies on the cell of origin. *Am J Surg Pathol* 1985;9:328-337.
46. Otto HF, Bettmann I, Weltzien Jv, Gebbers J-O. Primary intestinal lymphomas. *Virchows Arch* 1981;391:9-31.
47. MacLennan KA, Bennett MH, Tu A. The pathology of primary gastrointestinal lymphoma. *Clin Radiol* 1981;32:513-518.
48. Isaacson P, Wright DH, Judd MA, Mepham BL. Primary gastrointestinal lymphomas. A classification of 66 cases. *Cancer* 1979;43:1805-1819.
49. Lewin KJ, Ranchod M, Dorfman RF. Lymphomas of the gastrointestinal tract. A study of 117 cases presenting with gastrointestinal disease. *Cancer* 1978;42:693-707.
50. Van den Heule B, Van Kerkem C. Benign and malignant lymphoid lesions of the stomach. A histological reappraisal in the light of the Kiel classification for non-Hodgkin's lymphomas. *Histopathology* 1979;3:309-320.
51. Dutz W. The non-lymphomatous mucosa in primary gastrointestinal lymphoma: an etiological clue ? In XIIth Congress of the International Academy of Pathology and IIIrd World Congress of Academic and Environmental Pathology. Jerusalem, Israel, 1978:10-15.
52. Isaacson P. Primary gastrointestinal lymphoma. *Virchows Arch* 1981;391:1-8.
53. Isaacson P, Wright DH. Malignant lymphoma of mucosa-associated lymphoid tissue. (A distinctive type of B-cell lymphoma) *Cancer* 1983;52:1410-1416.
54. Papadaki L, Wotherspoon AC, Isaacson PG. The lymphoepithelial lesion of gastric low-grade B-cell lymphoma of mucosa-associated lymphoid tissue (MALT): an ultrastructural study. *Histopathology* 1992;21:415-421.
55. Li G, Hansmann ML, Zwingers T, Lennert K. Primary lymphomas of the lung: morphological, immunohistochemical and clinical features. *Histopathology* 1990;16:519-533.
56. Isaacson PG, Wotherspoon AC, Diss T, Pan L. Follicular colonization in B-cell lymphoma of mucosa-associated lymphoid tissue. *Am J Surg Pathol* 1991;15:819-828.

57. Cammoun M, Jaafoura H, Tabbane F, Halphen M. Immunoproliferative small intestinal disease without alpha-chain disease: a pathological study. *Gastroenterology* 1989;96:750-763.
58. Spencer J, Diss TC, Isaacson PG. Primary B-cell Gastric Lymphoma and 'pseudolymphoma': a genotypic study. *Am J Pathol* 1989;135:557-64.
59. Isaacson PG, Spencer J. Malignant lymphoma of mucosa-associated lymphoid tissue. *Histopathology* 1987;11:445-462.
60. Isaacson PG. Lymphomas of mucosa-associated lymphoid tissue (MALT). *Histopathology* 1990;16:617-619.
61. Myhre MJ, Isaacson PG. Primary B-cell gastric lymphoma - a reassessment of its histogenesis. *J Pathol* 1987;152:1-11.
62. Hall PA, Levison DA. Malignant lymphoma in the gastrointestinal tract. *Semin Diagn Pathol* 1991;8:163-177.
63. Laissue JA, Chappuis BB, Müller C, Reubi JC, Gebbers JO. The intestinal immune system and its relation to disease. *Digest Dis* 1993;11:298-312.
64. Cornes JS. Number, size and distribution of Peyer's patches in the human small intestine. Part II. The effect of age on Peyer's patches. *Gut* 1965;6:230.
65. Owen RL, Jones AL. Epithelial cell specialization within human Peyer's patches: an ultrastructural study of intestinal lymphoid follicles. *Gastroenterology* 1974;66:189.
66. Van Den Oord JJ, De Wolf-Peeters C, Desmet VJ. The marginal zone in the human reactive lymph node. *Am J Clin Pathol* 1986;86:475-479.
67. Spencer J, Finn T, Isaacson PG. Gut associated lymphoid tissue: a morphological and immunocytochemical study of the human appendix. *Gut* 1985;26:672-679.
68. Spencer J, Finn T, Pulford KAF, Mason DY, Isaacson PG. The human gut contains a novel population of B lymphocytes which resemble marginal zone cells. *Clin Exp Immunol* 1985;62:607-612.
69. MacLennan ICM, Gray D, Kumararatne DS, Bazin H. The lymphocytes of splenic marginal zones: a distinct B-cell lineage. *Immunol Today* 1982;3:305-307.
70. Chan JKC, Ng CS, Isaacson PG. Relationship between high-grade lymphoma and low-grade B-cell mucosa-associated lymphoid tissue lymphoma (MALToma) of the stomach. *Am J Pathol* 1990;136:1153-1163.
71. Montalbán C, Manzanal A, Castrillo JM, Escribano L, Bellas C. Low grade gastric B-cell MALT lymphoma progressing into high grade lymphoma. Clonal identity of the two stages of the tumour, unusual bone involvement and leukaemic dissemination. *Histopathology* 1995;27:89-91.
72. Peng H, Du M, Diss TC, Isaacson PG, Pan L. Genetic evidence for a clonal link between low and high-grade components in gastric MALT B-cell lymphoma. *Histopathology* 1997;30:425-429.
73. VanKrieken JHJM, Otter R, Hermans J, Van Groningen K, Spaander PJ, Van de Sandt MM, Keuning JF, Kluin PhM. Malignant lymphoma of the gastrointestinal tract and mesentery. *Am J Pathol* 1989;135:281-289.

74. Rohatiner A, D'Amore F, Coiffier B, Crowther D, Gospodarowicz M, Isaacsón P, Lister TA, Norton A, Salem P, Shipp M, Somers R. Report on a workshop convened to discuss the pathological and staging classifications of gastrointestinal tract lymphoma. *Ann Oncol* 1994;5:397-400.
75. Isaacson PG, Spencer J, Wright DH. Classifying primary gut lymphomas. *Lancet* 1988;2:1148-1149.
76. Ming S. Adenocarcinoma and other malignant tumours of the stomach. In: Ming SC, Goldman H, ed. *Pathology of the gastrointestinal tract*. Philadelphia: WB Saunders, 1992:584-618.
77. Warren JR. Unidentified curved bacilli on gastric epithelium in active chronic gastritis. *Lancet* 1983;1:1273.
78. Marshall B. Unidentified curved bacilli on gastric epithelium in active chronic gastritis. *Lancet* 1983;1:1273-1275.
79. Loffeld RJLF, Arends JW. The role of *Helicobacter pylori* in non-ulcer dyspepsia and gastritis. *Neth J Med* 1993;42:73-79.
80. Moss S, Calam J. *Helicobacter pylori* and peptic ulcers: the present position. *Gut* 1992;33:289-292.
81. Nomura A, Stemmerman GN. *Helicobacter pylori* and gastric cancer. *J Gastroen Hepatol* 1993;8:294-303.
82. Pajares JM. *H. pylori* infection: its role in chronic gastritis, carcinoma and peptic ulcer. *Hepato-Gastroenterol* 1995;42:827-841.
83. Parsonnet J, Friedman GD, Vandersteen DP. *Helicobacter pylori* and the risk of gastric carcinoma. *N Engl J Med* 1991;325:1127-1131.
84. Stark RM, Greenman J, Millar MR. Physiology and biochemistry of *Helicobacter pylori*. *Brit J Biomed Sci* 1995;52:282-290.
85. Calam J. *Helicobacter pylori*. *Eur J Clin Invest* 1994;24:501-510.
86. Fennerty MB. *Helicobacter pylori*. *Arch Intern Med* 1994;154:721-727.
87. Rautelin H, Kosunen TU. *Helicobacter pylori* and associated gastroduodenal disease. *APMIS* 1991;99:677-695.
88. The EUROGAST Study Group. Epidemiology of, and risk factors for, *Helicobacter pylori* infection among 3194 asymptomatic subjects in 17 populations. *Gut* 1993;34:1672-1676.
89. Cullen DJE, Collins BJ, Christiansen KJ, Epis J, Warren JR, Surveyor I, Cullen K. When is *Helicobacter pylori* infection acquired ? *Gut* 1993;34:1681-1682.
90. Wyatt JJ. Histopathology of gastroduodenal inflammation: the impact of *Helicobacter pylori*. *Histopathology* 1995;26:1-15.
91. Kozol RA, Dekhne N. *Helicobacter pylori* and the pathogenesis of duodenal ulcer. *J Lab Clin Med* 1994;124:623-6.
92. Dekigai H, Murkami M, Toru K. Mechanisms of *Helicobacter pylori*-associated gastric mucosal injury. *Digest Dis Sci* 1995;40:1332-1339.
93. Genta RM, Graham DY. *Helicobacter pylori*: the new bug on the paraffin block. *Virchows Arch* 1994;425:339-347.

94. Loffeld RJLF, Stobberingh E, Flendrig JA, Arends JW. *Helicobacter pylori* in gastric biopsy specimens. Comparison of culture, modified Giemsa stain, and immunohistochemistry. A retrospective study. *J Pathol* 1991;165:69-73.
95. Parsonnet J, Hansen S, Rodriguez L, Gelb A, Warnke RA, Jellum E, Orentreich N, Vogelmann JH, Friedman GD. *Helicobacter pylori* infection and gastric lymphoma. *N Engl J Med* 1994;330:1267-1271.
96. Genta RM, Hamner HW, Graham DY. Gastric lymphoid follicles in *Helicobacter pylori* infection: frequency, distribution, and response to triple therapy. *Hum Pathol* 1993;24:577-583.
97. Zaitoun AM. The prevalence of lymphoid follicles in *Helicobacter pylori* associated gastritis in patients with ulcers and non-ulcer dyspepsia. *J Clin Pathol* 1995;48:325-329.
98. Eidt S, Stolte M. Prevalence of lymphoid follicles and aggregates in *Helicobacter pylori* gastritis in antral and body mucosa. *J Clin Pathol* 1993;46:832-835.
99. Wotherspoon AC, Ortiz-Hidalgo C, Falzon MR, Isaacson PG. *Helicobacter pylori*-associated gastritis and primary B-cell gastric lymphoma. *Lancet* 1991;338:1175-1176.
100. Miettinen A, Karttunen TJ, Alavaikko M. Lymphocytic gastritis and *Helicobacter pylori* infection in gastric lymphoma. *Gut* 1995;37:471-476.
101. Wotherspoon AC, Doglioni C, Diss TC, Pan L, Moschini A, Boni M, Isaacson PG. Regression of primary low-grade B-cell gastric lymphoma of mucosa-associated lymphoid tissue type after eradication of *Helicobacter pylori*. *Lancet* 1993;342:575-577.
102. Roggero E, Zucca E, Pinotti G, Pascarella A, Capella C, Savio A, Pedrinis E, Paterlini E, Venco A, Cavalli F. Eradication of *Helicobacter pylori* infection in primary low-grade gastric lymphoma of mucosa-associated lymphoid tissue. *Ann Intern Med* 1995;122:767-769.
103. Weber DM, Dimopoulos MA, Anandu DP, Pugh WC, Steinbach G. Regression of gastric lymphoma of mucosa-associated lymphoid tissue with antibiotic therapy for *Helicobacter pylori*. *Gastroenterology* 1994;107:1835-1838.
104. Carlson SJ, Yokoo H, Vanagunas A. Progression of gastritis to monoclonal B-cell lymphoma with resolution and recurrence following eradication of *Helicobacter pylori*. *J Am Med Assoc* 1996;275:937-939.
105. Boot H, DeJong D, VanHeerde P, Taal B. Role of *Helicobacter pylori* eradication in high grade MALT lymphoma. *Lancet* 1995;346:448-449.
106. Calvert R, Randerson J, Evans P, Cawkwell L, Lewis F, Dixon MF, Jack A, Owen R, Shiach C, Morgan GJ. Genetic abnormalities during transition from *Helicobacter-pylori*-associated gastritis to low-grade MALToma. *Lancet* 1995;345:26-27.
107. Karat D, O'Hanlon DM, Hayes N, Scott D, Rames SA, Griffin SM. Prospective study of *Helicobacter pylori* infection in primary gastric lymphoma. *Brit J Surg* 1995;82:1369-1370.
108. Isaacson PG. Gastric lymphoma and *Helicobacter pylori*. *New Engl J Med* 1994;330:1310-1311.

109. Eidt S, Bayerdörffer E, Stolte M. Treat the infection and cure the cancer. *Lancet* 1995;345:874-875.
110. Vineis P, Italy Wgoteohmi. Incidence and time trends for lymphomas, leukemias and myelomas: hypothesis generation. *Leukemia Res* 1996;20:285-290.
111. Bailar JC, Gornik HL. Cancer undefeated. *New Engl J Med* 1997;336:1569-1574.
112. Morgan G, Vornanen M, Puitinen J, Naukkarinen A, Brincker H, Olsen J, Coeburgh JW, Vrints LWMA, Clayden D, McNally R, Jack A, Carli PM, Petrella T, Tommi R, D'Lollo S, Barchielli A, Cartwright R. Changing trends in the incidence of non-Hodgkin's lymphoma in Europe. *Ann Oncol* 1997;8:S49-S54.
113. Sandler RS. Primary gastric lymphoma: a review. *Am J Gastroenterol* 1984;79:21-25.
114. Hayes J, Dunn E. Has the incidence of primary gastric lymphoma increased ? *Cancer* 1989;63:2073-2076.
115. Severson RK, Davis S. Increasing incidence of primary gastric lymphoma. *Cancer* 1990;66:1283-1287.
116. Banfi A, Bonadonna G, Carnevali G, Oldini C, Salisni E. Preferential sites of involvement and spread in malignant lymphomas. *Eur J Cancer Clin Oncol* 1968;4:319-324.
117. Freeman C, Berg JW, Cutler SJ. Occurrence and prognosis of extranodal lymphomas. *Cancer* 1972;29:252-260.
118. Rudders RA, Ross ME, DeLellis RA. Primary extranodal lymphoma. Response to treatment and factors influencing prognosis. *Cancer* 1978;42:406-416.
119. D'Amore F, Christensen BE, Brincker H, Pedersen NT, Thorling K, Hastrup J, Pedersen M, Jensen MK, Johansen P, Andersen E, Bach B, Sorensen E, on behalf of the Danish LYFO Study Group. Clinicopathological features and prognostic factors in extranodal non-Hodgkin's lymphomas. *Eur J Cancer* 1991;27:1201-1208.
120. Allen AW, Donaldson G, Sniffen RC, Goodale F. Primary malignant lymphoma of the gastro-intestinal tract. *Ann Surg* 1954;140:428-438.
121. Bäck H, Gustavsson B, Ridell B. Primary gastrointestinal lymphoma: incidence, clinical presentation, and surgical approach. *J Surg Oncol* 1986;33:234-238.
122. Herrmann R, Panahon AM, Barcos M. Gastrointestinal involvement in non-Hodgkin's lymphoma. *Cancer* 1980;46:215-222.
123. Loehr WJ, Mujahed Z, Zahn FD, Gray GF, Thorbjarnarson B. Primary lymphoma of the gastrointestinal tract. A review of 100 cases. *Ann Surg* 1969;170:232-238.
124. O'Connor GT. Geographical variations in the occurrence of leukemias and lymphomas: summary and comments. In: Magrath J, O'Connor GT, Ramot B, ed. *Progress in Cancer Research and Therapy*. New York: Raven Press, 1984;27:123-27.
125. Salem P, El-Hashimi L, Anaissie E. Primary small intestinal lymphoma in adults: A comparative study of IPSID versus non-IPSID in the Middle East. *Cancer* 1987;59:1670-1676.
126. Doglioni C, Wotherspoon AC, Moschini A. High incidence of primary gastric lymphoma in northeastern Italy. *Lancet* 1992;339:834-835.

127. Dragosics B, Bauer P, Radaszkiewicz T. Primary gastrointestinal non-Hodgkin's lymphomas: a retrospective clinicopathological study of 150 cases. *Cancer* 1985;55:1060-1073.
128. Weingrad DN, Decosse JJ, Sherlock P, Straus D, Lieberman PH, Filippa DA. Primary gastrointestinal lymphoma: A 30-year review. *Cancer* 1982;49:1258-1265.
129. Weiss NS, Yang CP. Incidence of histologic types of cancer of the small intestine. *J Natl Cancer I* 1987;78:653-656.
130. DiSario JA, Burt RW, Kendrick ML, McWhorter WP. Colorectal cancers of rare histologic types compared with adenocarcinomas. *Dis Colon Rectum* 1994;37:1277-1280.
131. Zigelboim J, Larson MV. Primary colonic lymphoma. Clinical presentation, histopathological features, and outcome with combination chemotherapy. *J Clin Gastroenterol* 1994;18:291-297.
132. Levine AM. Acquired immunodeficiency syndrome-related lymphoma. *Blood* 1992;80:8-20.
133. Nalesnik MA, Jaffe R, Starzl TE. The pathology of posttransplant lymphoproliferative disorders occurring in the setting of cyclosporine A-prednisone immunosuppression. *Am J Pathol* 1988;133:173-192.
134. Rosenberg SA. Validity of the Ann Arbor Staging Classification for the non-Hodgkin's lymphomas. *Cancer Treat Rep* 1977;61:1023-1027.
135. Rosenberg SA. Report of the committee on the staging of Hodgkin's disease. *Cancer Res* 1966;26:1042-1311.
136. Arbor A. Symposium 'Staging in Hodgkin's disease'. *Cancer Res* 1971;31:1707-1870.
137. Peters WV, Bush RS, Brown TC, Reid J. The place of radiotherapy in the control of non-Hodgkin's lymphomas. *Brit J Cancer* 1975;31:386-401.
138. Musshoff K. Prognostic and therapeutic implications of staging in extranodal Hodgkin's disease. *Cancer Res* 1971;31:1814-1827.
139. Musshoff K, Schmidt-Vollmer H. Prognosis of non-Hodgkin's lymphomas with special emphasis on the staging classification. *Z Krebsforschung* 1975;83:323-341.
140. Musshoff K. Klinische Stadieneinteilung der Nicht-Hodgkin-Lymphome. *Strahlentherapie* 1977;153:218-222.
141. Blackledge G, Best JK, Crowther D. Role of computed tomography in staging and management of gastrointestinal lymphoma. *J Roy Soc Med* 1979;72:818-822.
142. Shimodaira M, Tsukamoto Y, Niwa Y, Goto H, Hase S, Hayakawa T, Nagasaka T. A proposed staging system for primary gastric lymphoma. *Cancer* 1994;73:2709-2715.
143. Liang R, Todd D, Chan TK, Chiu E, Lie A, Kwong YL, Choy D, Ho FCS. Prognostic factors for primary gastrointestinal lymphoma. *Hematol Oncol* 1995;13:153-163.
144. Naqvi MS, Burrows L, Kark AE. Lymphoma of the gastrointestinal tract: prognostic guides based on 162 cases. *Ann Surg* 1969;170:221-231.

145. Azab MB, Henry-Amar M, Rougier P, Bognel C, Theodore C, Carde P, Lasser P, Losset JM, Caillou B, Droz J-P, Hayat M. Prognostic factors in primary gastro-intestinal non-Hodgkin's lymphoma. *Cancer* 1989;64:1208-1217.
146. Otter R, Bieger P, Hermans J, Willemze R. Primary gastrointestinal non-Hodgkin's lymphoma in a population-based registry. *Brit J Cancer* 1989;60:745-50.
147. List AF, Greer JP, Cousar JC, Stein RS, Johnson DH, Reynolds VH, Greco FA, Flexner JM, Hande KR. Non-Hodgkin's lymphoma of the gastrointestinal tract: an analysis of clinical and pathologic features affecting outcome. *J Clin Oncol* 1988;6:1125-1133.
148. Jaser N, Sivula A, Franssila K. Primary gastric non-Hodgkin's lymphoma in Finland, 1972-1977. Clinical presentation and results of treatment. *Scand J Gastroenterol* 1990;25:1052-1059.
149. Cogliatti SB, Schmid U, Schumacher U. Primary B-cell gastric lymphoma: a clinicopathological study of 145 patients. *Gastroenterology* 1991;64:1159-1170.
150. Radaszkiewicz T, Dragosics B, Bauer P. Gastrointestinal malignant lymphomas of mucosa-associated lymphoid tissues: factors relevant to prognosis. *Gastroenterology* 1992;102:1628-1638.
151. Montalbán C, Castrillo JM, Abaira V, Serrano M, Bellas C, Piris MA, Carrion R, Cruz MA, Larana JG, Menauguez J, Gomez MF, Rivas C. Gastric B-cell mucosa-associated lymphoid tissue (MALT) lymphoma. Clinicopathological study and evaluation of the prognostic factors in 143 patients. *Ann Oncol* 1995;6:355-362.
152. Taal BG, Burgers JMV, VanHeerde P, Hart AAM, Somers R. The clinical spectrum and treatment of primary non-Hodgkin's lymphoma of the stomach. *Ann Oncol* 1993;4:839-846.
153. Taal BG, Boot H, VanHeerde P, DeJong D, Hart AAM, Burgers JMV. Primary non-Hodgkin lymphoma of the stomach: endoscopic pattern and prognosis in low versus high grade malignancy in relation to the MALT concept. *Gut* 1996;39:556-561.
154. Hockey PG, Powell J, Crocker J, Fielding JW. Primary gastric lymphoma. *Brit J Surg* 1987;74:483-487.
155. Rosen CB, VanHeerden JA, Martin JK, Wold LE, Ilstrup DM. Is an aggressive surgical approach to the patient with gastric lymphoma warranted ? *Ann Surg* 1987;205:634-640.
156. Shiu MH, Nisce LZ, Pinna A, Straus DJ, Tome M, Filippa DA. Recent results of multimodal therapy of gastric lymphoma. *Cancer* 1986;58:1389-1399.
157. Burgers JM, Taal BG, Van Heerde P, Somers R, DenHartog-Jager FC, Hart AA. Treatment results of primary stage I and II non-Hodgkin's lymphoma of the stomach. *Radiotherapy Oncol* 1989;11:319-326.
158. Maor MH, Maddux B, Osborne BM, Fuller LM, Sullivan JA, Nelson SR. Stages IE and IIE non-Hodgkin's lymphomas of the stomach. Comparison of treatment modalities. *Cancer* 1984;54:2330-2337.
159. Gobbi PG, Dionigi P, Barbieri F, Corbella F, Bertoloni D, Grignani G, Jemos V, Pieresca C, Ascari E. The role of surgery in the multimodal treatment of primary gastric non-Hodgkin's lymphomas. *Cancer* 1990;65:2528-2536.

160. Salles G, Herbrecht R, Tilly H, Berger F, Brousse N, Gisselbrecht C. Aggressive primary gastrointestinal lymphomas: review of 91 patients treated with the LNH-84 regimen. A study of the Groupe d'Etude des Lymphomes Agressifs. *Am J Med* 1991;90:77-84.
161. Bayerdorffer E, Neubauer A, Rudolph B, Thiede C, Lehn N, Eidt S, Stolte M. Regression of primary gastric lymphoma of mucosa-associated lymphoid tissue after cure of *Helicobacter pylori* infection. MALT Lymphoma Study Group. *Lancet* 1995;345:1591-1594.
162. Alkan S, Karcher DS, Newman MA, Cohen P. Regression of salivary gland MALT lymphoma after treatment for *Helicobacter pylori*. *Lancet* 1996;348:268-269.
163. Fischbach W, Tacke W, Greiner A, Konrad H, Müller-Hermelink M. Regression of immunoproliferative small intestinal disease after eradication of *Helicobacter pylori*. *Lancet* 1997;349:31-32.
164. Matsumoto T, Iida M, Shimizu M. Regression of mucosa-associated lymphoid-tissue lymphoma of rectum after eradication of *Helicobacter pylori*. *Lancet* 1997;350:115-116.
165. Nagashima R, Takeda H, Maeda K, Ohno S, Takahashi T. Regression of duodenal mucosa-associated lymphoid tissue lymphoma after eradication of *Helicobacter pylori*. *Gastroenterology* 1996;111:1674-1678.
166. Morgan DR, Holgate CS, Dixon MF, Bird CC. Primary small intestinal lymphoma: a study of 39 cases. *J Pathol* 1985;147:211-221.
167. Jaser N, Sivula A. Primary small intestinal non-Hodgkin's lymphoma in Finland 1972-1977. *Ann Chir Gynaecol* 1991;80:250-257.
168. Rubesin SE, Gilchrist AM, Bronner M, Saul SH, Herlinger H, Grumbach K, Levine MS, Laufer I. Non-Hodgkin lymphoma of the small intestine. *RadioGraphics* 1990;10:985-998.
169. Kayabali I, Gökcora IH, Bacaci K, Cavusoglu T, Kutlu K. Primary colonic non-Hodgkin's lymphomas: A retrospective clinicopathological study of 14 cases. *Int Surg* 1992;77:195-197.
170. Aozasa K, Ohsawa M, Soma T, Sugiyama H, Yano Y, Shimano T, Hara K, Yamashita I, Ito M, Kuze S. Malignant lymphoma of the rectum. *Jpn J Clin Oncol* 1990;20:380-386.
171. Busch E, Rodriguez-Bigas M, Mamounas E, Barcos M, Petrelli NJ. Primary colorectal non-Hodgkin's lymphoma. *Ann Surg Oncol* 1994;1:222-228.
172. Ruskoné-Fourmestiaux A, Delmer A, Lavergne A, Molina T, Brousse N, Audouin J, Rambaud JC. Multiple lymphomatous polyposis of the gastrointestinal tract: prospective clinicopathological study of 31 cases. *Gastroenterology* 1997;112:7-16.
173. Fraga M, Lloret E, Sanchez-Verde L, Orradre JL, Campo E, Bosch F, Piris MA. Mucosal mantle cell (centrocytic) lymphomas. *Histopathology* 1995;26:413-422.
174. Pan L, Diss TC, Peng H, Wotherspoon AC, Thomas JA, Isaacson PG. Epstein-Barr virus (EBV) in enteropathy-associated T-cell lymphoma (EATL). *J Pathol* 1993;170:137-143.

175. Ilyas M, Niedobitek G, Aganthatgelou A, Barry RE, Read AE, Tierney R, Young LS, Rooney N. Non-Hodgkin's lymphoma, coeliac disease, and epstein-barr virus: a study of 13 cases of enteropathy-associated T- and B-cell lymphoma. *J Pathol* 1995;177:115-122.
176. Murray A, Cuevas EC, Jones DB, Wright DH. Study of the immunohistochemistry and T cell clonality of Enteropathy-associated T cell lymphoma. *Am J Pathol* 1995;146:509-519.
177. Price SK. Immunoproliferative small intestinal disease: A study of 13 cases with alpha heavy-chain disease. *Histopathology* 1990;17:7-17.
178. Khojasteh AK, Haghighi P. Immunoproliferative small intestinal disease: portrait of a potentially preventable cancer from the Third World. *Am J Med* 1990;89:483-490.
179. Turowski GA, Basson MD. Primary malignant lymphoma of the intestine. *Am J Surg* 1995;169:433-441.
180. Heise W, Dederke B, Riecken EO. Intestinale non-Hodgkin-Lymphome. *Dtsch Med Wschr* 1995;120:1447-1451.
181. Czader M, Mazur J, Pettersson M, Liliemark J, Stromberg M, Christensson B, Tribukait B, Aner G, Ost A, Pouwit A. Prognostic significance of proliferative and apoptotic fractions in low grade follicle center cell-derived non-Hodgkin's lymphomas. *Cancer* 1996;77:1180-1188.
182. Korkolopoulou P, Oates J, Kittas C, Crocker J. P53,c-myc p62 and proliferating cell nuclear antigen (PCNA) expression in non-Hodgkin's lymphomas. *J Clin Pathol* 1994;47:9-14.
183. Grogan TM, Lippman SM, Spier CM, Slymen DJ, Rybski JA, Rangel CS. Independent prognostic significance of a nuclear proliferation antigen in diffuse large cell lymphomas as determined by the monoclonal antibody Ki-67. *Blood* 1988;71:1157-1160.
184. Medeiros LJ, Picker LJ, Gelb AB, Strickler JG, Brain SW, Weiss LM. Numbers of host "helper" T-cells and proliferating cells predict survival in diffuse small-cell lymphomas. *J Clin Oncol* 1989;7:1009-1017.
185. Spina D, Leoncini L, Del Vecchio MT, Megha T, Minacci C, Poggi SA, Pileri SA, Tosi P, Kraft R, Laissue JA, Cottier H. Low versus high cell turnover in diffusely growing non-Hodgkin's lymphomas. *J Pathol* 1995;177:335-341.
186. Fischbach W, Böhm S. *Helicobacter-pylori*-assoziierte Gastritis und primäres magenlymphom. *Z Gastroenterol* 1993;31:327-328.
187. Isaacson PG, Spencer J. Is gastric lymphoma an infectious disease ? *Hum Pathol* 1994;24:569-570.
188. Wotherspoon AC, Finn TM, Isaacson PG. Trisomy 3 in low-grade B-cell lymphomas of mucosa-associated lymphoid tissue. *Blood* 1995;85:2000-2004.

Chapter 2

Materials and Methods

The Limburg Working Party on Lymphoma

Design and population

The Limburg Working Party on Lymphoma was established in 1986. Participators were pathologists of the University Hospital Maastricht, De Wever Hospital Heerlen, St. Gregorius Hospital Brunssum, St. Jozef Hospital Kerkrade, Maasland Hospital in Sittard & Geleen, St. Laurentius Hospital Roermond (combined laboratory of pathology for Roermond and Weert), St. Maartens Gasthuis Venlo (combined laboratory for Venlo and Venray). The purpose of the Party was to review all new cases of suspected lymphoma in order to make a more accurate decision and to share knowledge about these lymphoproliferative disorders. For this purpose, sections of every new case, diagnosed in one of these hospitals were sent to all participating pathologists, to give them the opportunity to study and classify the lesion. Monthly, all cases were reviewed and discussed plenary in order to reach consensus about the diagnosis. The diagnosis of the panel was not primarily meant for clinical purposes.

The population covered by these ten hospitals is the province of Limburg, which has 1.133.669 inhabitants (on January, 1 1996).

Selection of cases from the registry

Time period

On the basis of the registry of the Limburg Working Party on Lymphoma, which consists of a list of patients, their birth dates, treating hospital, localisation of the lesion and consensus diagnosis it was possible to select consecutive cases of primary gastro-intestinal cases. We choose to study the cases from the start of the Working Party in July, 1986 until December, 1994 in order to make the clinical follow-up time at least one year. The last general follow-up round in the hospitals was performed in December 1995 and January 1996, and only in cases with missing data on survival, the general practitioner was asked for additional information, in the course of 1997.

Selection

The registry was searched for cases of gastro-intestinal non-Hodgkin's lymphoma: all cases with a suspected or proven diagnosis of non-Hodgkin's lymphoma presenting in the gastrointestinal tract (including: stomach, duodenum, jejunum, ileum, colon, appendix, rectum, anal canal, as well as mesenteric lymph nodes) were selected, without further knowledge about clinical data.

After this first rough selection, paraffin-embedded tissue blocks, as well as definitive pathology reports were collected from the pathology files of the participating hospitals, in order to review the cases histologically. When tissue blocks could not be obtained, these cases could not be used for the immunohistochemical and molecular biological part of this study, but in order to keep the population based series as complete as possible (at least the clinical data), slides from these cases were obtained from the files of the Working Party on Lymphoma and reviewed.

Furthermore, clinical data were collected from the archives of the participating hospitals, in cooperation with the treating clinicians. When no clinical data at all could be obtained, cases were excluded from this study.

Pathological selection criteria, review of the material

From the paraffin-embedded tissue blocks, 2 μ m sections were cut and stained using a routine haematoxylin-eosin stain, in order to revise the material. As mentioned before, when no tissue blocks could be obtained, slides previously used by the Working Party were revised.

All slides were revised and assessed initially for their association with the mucosa. Cases which only showed mesenterial lymphoma, without involvement of the mucosa of the gastrointestinal tract were excluded.

Classification of the cases was performed by two pathologists (JW Arends, FJ Bot). All cases were classified according to the proposal made during the Vth International Conference on Malignant lymphoma, in Lugano, Switzerland 1993¹. This is a classification for gastrointestinal lymphomas, based on the principles of the Kiel classification. Briefly, a major distinction is made between B- and T-cell lymphomas, and between small and large cell cases. Cases are furthermore classified as MALT lymphoma when the typical features of MALT are present. Large cell MALT lymphomas are diagnosed when sheets of large blastic cells are present and there is evidence of a small cell MALT component in the same lymphoma. When the diagnosis MALT lymphoma cannot be made, cases can be classified according to one of the categories of their nodal equivalents. Patients with Burkitt's lymphoma were excluded from this study, because most affected patients were children and because of the specific pathogenesis of Burkitt's lymphoma^{2,3}.

Clinical selection criteria

From the clinical and outpatient files, the data summarized in table 2.1 were recorded. In first instance, data were recorded for each case. Afterwards, these data were examined to establish whether the lymphomas diagnosed in the gastrointestinal tract indeed were primary gastrointestinal lymphomas. To meet with this criterion the gastrointestinal tract lesion had to be the only manifestation of

lymphoma or the gastrointestinal tract had to be the most massively affected organ whereas there should be no evidence of former or concurrent primary nodal disease. When data were insufficient to assess so, cases were excluded.

Table 2.1 Clinical data.

Categories	data
Patient identification	date of birth, age, sex, race
Signs and symptoms at presentation	night sweats, weight loss, fever, anorexia, vomiting, nausea, diarrhoea, gastrointestinal bleeding, dysphagia, obstructions, perforations, dyspeptic complaints, pain
Physical examination	Lymphadenopathy, spleen and/or liver enlargement
Laboratory investigations	LDH, Haemoglobin, white blood cell/platelet counts, albumin, total protein
Medical history	Peptic ulcer disease, villous atrophy, malabsorption, coeliac disease, Crohn's disease, ulcerative colitis, abdominal surgery
Staging procedures	CT scan (lymph nodes, spleen, liver involvement), bone marrow involvement, Fluorescence Activated Cell Sorting, X-Thorax (mediastinal lymph nodes), Ear, Nose & Throat examination
Surgical therapy	date, resection site, involvement of resection planes and regional lymph nodes, complications
Radiotherapy	date, radiation field, dose, complications
Chemotherapy	regimen, number of cycles, complications
Patient status	Remission (no/partial/total), date, recurrent disease (date and localisation), last follow up, death with or without disease, cause of death.

In summary, after the first rough selection from the registry of the Limburg Working Party on Lymphoma, 143 cases were selected. From these cases we had to exclude 26 cases for the following reasons: concurrent or former nodal disease ($n=7$); Burkitt's lymphoma ($n=4$); mesenterial lymph nodes ($n=2$); patients from Belgium ($n=6$); reactive lymph node in revision ($n=1$); adenocarcinoma in revision ($n=1$); linitis plastica in revision ($n=1$); morbus Richter ($n=1$); CLL ($n=1$); recurrent disease in earlier included patients ($n=2$).

Using the criteria described above 117 cases could be considered as primary gastrointestinal non-Hodgkin's lymphoma and were evaluable for further investigation. However, from eight of these patients adequate follow-up data are missing, excluding them from the retrospective clinical studies.

Features of selected cases

Selected cases per year

The total number of 117 primary gastrointestinal lymphomas is 8.3% of the total number of diagnosed lymphomas (n=1362) in this time period. This is in accordance with earlier reports on the occurrence of primary gastrointestinal lymphomas among non-Hodgkin's lymphomas.

Figure 2.1 shows the number of primary gastrointestinal non-Hodgkin's lymphomas diagnosed each year in the time period from 1986 till 1994. As can be noted from this figure, there is no absolute increase or decrease in the percentage of primary gastro-intestinal cases.

Selected cases per hospital

In figure 2.2, the number of cases, diagnosed in the participating hospitals is shown. As can be noticed from the figure, most cases were diagnosed in University Hospital Maastricht.

Localisation in the gastrointestinal tract

In most studies dealing with primary gastrointestinal lymphomas, the stomach turns out to be the site most often affected. We analyzed the site of origin of our cases (figure 2.3) and also found that the stomach is the commonest site for primary gastrointestinal lymphoma.

Age and sex distribution

Of the 117 cases selected, 67 patients were male and 50 patients were female (M:F=1:0.75). Figure 2.4 shows the age distribution of the selected cases, with a mean age of 63 and a median age of 65 years.

Clinical stage at presentation

Figure 2.5 shows the percentages of small and large cell primary gastrointestinal lymphomas presenting in the different clinical stages. Stages were defined according to the modified Ann Arbor classification⁴.

Figure 2.1 Primary gastrointestinal non-Hodgkin's lymphoma from 1986 till 1994.

Figure 2.2 Distribution of cases in the participating hospitals.

Figure 2.3 Localisation of lymphomas throughout the gastrointestinal tract.

Figure 2.4 Age distribution of gastrointestinal lymphomas.

Figure 2.5 Stage at presentation in small and large B-cell lymphomas.

Pathological classification after revision

After revision of the material, and classification as described above, the distribution shown in table 2.2 was found. Small cell MALT lymphoma, Large cell MALT lymphoma and large cell lymphoma without MALT features were the three most common diagnoses, together accounting for 2/3 of the cases. Mantle cell lymphoma and T-cell lymphoma were not frequently found.

Table 2.2 Classification.

Histology	N	%
MALT, small	39	33.3
MALT, large	32	27.4
Large	35	29.9
T-small	3	2.6
T-large	6	5.1
Mantle cell	2	1.7

Methods

Cell proliferation and apoptosis

Cell proliferation has been studied extensively in human tumours, using several principles, like measurements of DNA synthesis in cell populations (f.e. 5-bromo-2-deoxy-uridine BrdU incorporation in the cellular DNA) and methods for studying cell viability and proliferation in single cells. The latter are mainly assays that monitor the expression of cell cycle-associated antigens, by which the number of cells in a certain phase of the cell cycle can be determined. For immuno-histochemical analysis several antibodies have been raised⁵, each with their own advantages and drawbacks. We used the mib-1 antibody, which reacts to Ki-67 and can be used in formalin-fixed routinely processed tissues^{6,7}. The Ki-67 antigen is expressed in all human proliferating cells and is preferentially expressed during the late G1, S, G2 and M phases of the cell cycle, while resting, non-cycling cells lack this antigen^{8,9}.

The balance between cell proliferation, cell differentiation and cell death determines the growth and the size of a tumour¹⁰⁻¹³. One of the major mechanisms of cell death is apoptosis, programmed cell death. Apoptosis has first been described by Wyllie *et al.* and since then has been characterized more and more¹⁴. The word apoptosis is derived from the Greek language and means 'dropping of leaves', to point out the physiological basis of it, in contrast to necrosis.

Apoptosis is characterized by a number of morphological and biochemical hallmarks, and moreover is subject to very strict regulatory processes: morphologically, the cell shrinks, the chromatin condenses and beads against the nuclear membrane, and apoptotic bodies are formed. In contrast to necrosis, no inflammatory response occurs and apoptotic bodies and apoptotic cells are phagocytosed very early by surrounding cells or phagocytes.

Biochemically, during apoptosis, chromatin is fragmented by endonucleases, in first instance in fragments of 200 kb and later by Mg²⁺ en Ca dependent endonucleases in nucleosome-sized fragments¹⁵⁻¹⁸.

Like proliferation, programmed cell death or apoptosis, is characterized by very

strict regulatory processes, in contrast to necrosis. A number of genes regulating apoptosis have been described and their mechanism of action is elucidated^{19,20}. We also studied the expression of two of these in relation to the rate of apoptosis: the *bcl-2* and the *p53* gene products. In experimental studies, it has been shown that the *bcl-2* gene, when expressed on a cell line, can inhibit apoptosis in these cells, leading to prolonged survival in these cells. These cells can therefore confer a multidrug resistance phenotype²¹⁻²⁴.

The *p53* tumour suppressor gene is able to induce a cell cycle arrest in the G1/S phase and the G2/M phase in response to DNA damage of the cell. During this cell cycle arrest the cell can repair its DNA in case of minor damage and in case of major damage undergoes apoptosis. If the *p53* gene is mutated, this cell cycle checkpoint is lost and apoptosis is not induced by *p53* function²⁵⁻²⁸. Both the *bcl-2* gene product and the mutated *p53* protein can be detected by immunohistochemistry²⁹⁻³³.

Several methods have now been designed to detect apoptosis; the most well-known are the assays that measure DNA fragmentation, e.g. standard and pulsed agarose field gel electrophoresis, which shows a characteristic ladder-pattern as a result of the chromatin fragmentation.

For the detection of apoptosis at the single cell level in a tissue, methods also based on chromatin fragmentation have been designed: active labelling of cells by nick translation and active labelling of cells by end labelling (TUNEL)^{34,35}. In both procedures the free 3'-OH termini are labelled with modified nucleotides (labelled with fluorescein, biotin, or digoxigenin). The labelling enzyme used in the nick translation is DNA polymerase, in the TUNEL method, the enzyme terminal deoxynucleotidyl transferase is used. Because the latter is template independent, more label is introduced and therefore the TUNEL method should be more sensitive than the nick translation. Technically, residues of digoxigenin-nucleotide are catalytically added to the DNA with terminal deoxynucleotidyltransferase (TdT), an enzyme which causes addition of deoxyribonucleotide triphosphate to the 3'-OH ends of the DNA fragments. Second, the digoxigenin labelled dUTP is detected by an anti-digoxigenin antibody conjugated with peroxidase. This peroxidase enzyme generates an intense signal from chromogenic substrates.

In situ hybridization

Several methods to detect quantitative and structural genetic changes have been developed, such as flow cytometry, karyotyping and molecular techniques.

In flowcytometry (FCM) the total DNA content of a tumour cell population is quantifiable.^{36,37} Karyotyping can visualize chromosome structure and allows evaluation of individual chromosomes. DNA sequencing, Southern and Northern blotting, RFLP analysis and PCR are molecular techniques that can identify DNA,

RNA and protein changes. Drawbacks of these techniques are respectively, the inability to assess genetic changes in individual cells; the selection of clones with a high mitotic potential by culturing cells to obtain metaphase spreads; and again the inability to detect changes at the single cell level³⁸.

Interphase cytogenetics in the form of *in situ* hybridization (ISH) using chromosome specific DNA probes enables the detection of numerical chromosomal aberrations at the single cell level in interphase nuclei³⁹⁻⁴¹. *In situ* hybridisation was introduced by Cremer and colleagues in 1986⁴² and is based on the hybridisation of denaturated target DNA with denaturated probe DNA. In this study we used DNA probes recognizing several hundreds up to several thousands of higher order repeats, localized in the centromeric regions of the individual chromosomes to detect numerical chromosomal aberrations. These probes were labelled with biotin or digoxigenin to make them detectable. Several detection systems can be used, using either fluorochromes or enzymes conjugated with antibodies or avidin molecules. Furthermore, in this study, the catalyzed reporter deposition (CARD) system was used for amplification of the signals^{43,44}. In this system a (detectable) biotinylated tyramine is enzymatically (horseradish-peroxidase) precipitated around the original signal. Weak signals can in this way be intensified and easier evaluated.

Very important for the quality of the technique is the preparation of the biological material. Fixation in formaldehyde can cause crosslinking of nuclear proteins, making it necessary to use a proteolytic treatment to permeabilize the cell for the DNA probes and antibodies.

Evaluation of the technique is done by counting spots in a fixed number of nuclei assuming that the chromosome copy number is identical to the number of signals obtained by ISH with a specific centromeric probe⁴¹. For adequate evaluation strict criteria are used: nuclei should not overlap, the ISH signals should have more or less the same homogeneous fluorescence intensity, minor hybridisation signals should not be counted and spots in a paired arrangement (split spots), close to each other are counted as one spot⁴⁵. In this way ISH can be used as an important tool for the detection of numerical chromosomal aberrations.

References

1. Rohatiner A, D'Amore F, Coiffier B, Crowther D, Gospodarowicz M, Isaacson P, Lister TA, Norton A, Salem P, Shipp M, Somers R. Report on a workshop convened to discuss the pathological and staging classifications of gastrointestinal tract lymphoma. *Ann Oncol* 1994;5:397-400.
2. Magrath I. The pathogenesis of Burkitt's lymphoma. *Advances in Cancer Research* 1990;55:133-270.
3. Wright DH. What is Burkitt's lymphoma. *J Pathol* 1997;182:125-127.
4. Musshoff K. Klinische stadieneinteilung der nicht-Hodgkin Lymphome. *Strahlentherapie* 1977;153:218-221.
5. Yu CCW, Filipe MI. Update on proliferation-associated antibodies applicable to formalin-fixed paraffin-embedded tissue and their clinical applications. *Histochem J* 1993;25:843-853.
6. Key G, Becker MHG, Baron B, Duchrow M, Schlüter C, Flad HD. New Ki-67-equivalent murine monoclonal antibodies (MIB 1-3) generated against bacterially expressed parts of the Ki-67 cDNA containing three 62bp repetitive elements encoding for the Ki-67 epitope. *Lab Invest* 1993;68:629-636.
7. Gerdes J, Becker MHG, Key G, Cattoretti G. Immunohistochemical detection of tumour growth fraction (Ki-67 antigen) in formalin-fixed and routinely processed tissues. *J Pathol* 1992;168:85-87.
8. Key G, Larsen-Petersen J, Becker MHG, Duchrow M, Schlutter C, Askaa J, Gerdes J. New antiserum against Ki-67 antigen suitable for double immunostaining of paraffin wax sections. *J Clin Pathol* 1993;46:1080-1084.
9. Gerdes J, Lemke H, Baisch H, Wacker HH, Schwab U, Stein H. Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. *Journal of Immunology* 1984;
10. Marx J. Cell death studies yield cancer clues. *Science* 1993;259:760-761.
11. Kerr JFR, Winterford CM, Harmon BV. Apoptosis. Its significance in cancer and cancer therapy. *Cancer* 1994;73:2013-2026.
12. Spina D, Leoncini L, Del Vecchio MT, Megha T, Minacci C, Poggi SA, Pileri SA, Tosi P, Kraft R, Laissue JA, Cottier H. Low versus high cell turnover in diffusely growing non-Hodgkin's lymphomas. *J Pathol* 1995;177:335-341.
13. Sedivy R. The potential role of apoptosis (programmed cell death) in a chaotic determined carcinogenesis. *Medical Hypotheses* 1996;46:455-457.
14. Wyllie AH, Kerr JRR, Currie AR. Cell death: the significance of apoptosis. *Int Rev Cytol* 1980;68:251.
15. Vaux DL, Strasser A. The molecular biology of apoptosis. *Proc Natl Acad Sci USA* 1996;93:2239-2244.
16. Que FG, Gores GJ. Cell death by apoptosis: Basic concepts and disease relevance for the gastroenterologist. *Gastroenterology* 1996;110:1238-1243.
17. Lin XS, Denmeade SR, Isaacs JT. The genetics of programmed (apoptotic) cell death. *Cancer Surv* 1995;25:173-194.

18. Salomon RN, Diaz-Cano S. Introduction to apoptosis. *Diagn Mol Pathol* 1995;4:235-238.
19. Hoffman B, Liebermann DA. Molecular controls of apoptosis: differentiation/growth arrest primary response genes, proto-oncogenes, and tumor suppressor genes as positive and negative modulators. *Oncogene* 1994;9:1807-1812.
20. Hale AJ, Smith CA, Sutherland LC, Stoneman VEA, Longthorne VL, Culhane AC, Williams GT. Apoptosis: molecular regulation of cell death. *Eur J Biochem* 1996;236:1-26.
21. Hockenbery DM. The bcl-2 gene and apoptosis. *Sem Immunol* 1992;4:413-420.
22. Chao DT, Linette GP, Boise LH, White LS, Thompson CB, Korsmeyer SJ. Bcl-x_L and Bcl-2 repress a common pathway of cell death. *J Exp Med* 1995;182:821-828.
23. Farrow SN, Brown R. New members of the Bcl-2 family and their protein partners. *Curr Opin Gen Dev* 1996;6:45-49.
24. Kernohan NM, Cox LS. Regulation of apoptosis by bcl-2 and its regulated proteins: immunochemical challenges and their therapeutic implications. *J Pathol* 1996;179:1-3.
25. Agarwal ML, Agarwal A, Taylor WR, Stark GR. p53 controls both the G2/M and the G1 cell cycle checkpoints and mediates reversible growth arrest in human fibroblasts. *Proc Natl Acad Sci USA* 1995;92:8493-8497.
26. Bates S, Vousden KH. p53 in signaling checkpoint arrest or apoptosis. *Curr Opin Gen Dev* 1996;6:12-19.
27. Lane DP, Benchimol S. p53: oncogene or anti-oncogene? *Gene Dev* 1990;4:1-8.
28. Levine AJ, Momand J, Finlay CA. The p53 tumour suppressor gene. *Nature* 1991;351:453-456.
29. Baas IO, Mulder JWR, Offerhaus GJA, Vogelstein B, Hamilton SR. An evaluation of six antibodies for immunohistochemistry of mutant p53 gene product in archival colorectal neoplasms. *J Pathol* 1994;172:5-12.
30. Van De Berg FM, Baas IO, Polak MM, Offerhaus GJA. Detection of p53 overexpression in routinely paraffin-embedded tissue of human carcinomas using a novel target unmasking fluid. *Am J Pathol* 1993;142:381-385.
31. Hall PA, Lane DP. p53 in tumour pathology: can we trust immunohistochemistry? - revisited! *J Pathol* 1994;172:1-4.
32. Kerns BM, Jordan PA, Moore MBH, Humphrey PA, Berchuk A, Kohler MF, Bast RC, Iglehart D, Marks JR. p53 overexpression in formalin-fixed, paraffin-embedded tissue detected by immunohistochemistry. *J Histochem Cytochem* 1992;40:1047-1051.
33. Kondo E, Nakamura S, Onoue H, Matsuo Y, Yoshino T, Aoki H, Hayashi K, Takahashi K, Minowada J, Nomura S, Akagi T. Detection of bcl-2 protein and bcl-2 messenger RNA in normal and neoplastic lymphoid tissues by immunohistochemistry and in situ hybridization. *Blood* 1992;80:2044-2051.
34. Gavrieli Y, Sherman Y, Ben-Sasson SA. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J Cell Biol* 1992;119:493-501.

35. Wijsman JH, Jonker RR, Keijzer R, Van De Velde CJH, Cornelisse CJ, Van Dierendonck JH. A new method to detect apoptosis in paraffin sections: in situ end-labeling of fragmented DNA. *J Histochem Cytochem* 1993;41:7-12.
36. Merkel DE, Dressler LG, McGuire WL. Flow cytometry, cellular DNA content, and prognosis in human malignancy. *J Clin Oncol* 1987;5:1690-1703.
37. Barlogie B, Raber M, Schuman J, Johnson TS, Drewinko B, Swartzendruber DE, Gohde W, Andreeff M, Freireich EJ. Flow cytometry in clinical cancer research. *Cancer Res* 1983;43:3982-3997.
38. Poddighe PJ, Ramaekers FCS, Hopman AHN. Interphase cytogenetics of tumours. *J Pathol* 1992;166:215-224.
39. Hopman AHN, Raap AK, Landegent JE, Wiegant J, Boerman RH, VanDerPloeg M. Non-radioactive in situ hybridization. *Molec Neuroanat* 1988;1988:43-68.
40. Hopman AHN, Poddighe P, Moesker O, Ramaekers FCS. Interphase cytogenetics: an approach to the detection of genetic aberrations in tumours. *Diagn Mol Pathol* 1992;1:141-167.
41. Hopman HNH, Voorter CEM, Ramaekers FCS. Detection of genomic changes in cancer by in situ hybridization. *Mol Biol Rep* 1994;19:31-44.
42. Cremer T, Landegent J, Brückner A, Scholl HP, Schardin M, Hager HD, Devilee P, Pearson P, Van Der Ploeg M. Detection of chromosome aberrations in the human interphase nucleus by visualization of specific target DNAs with radioactive and non-radioactive in situ hybridization methods: diagnosis of trisomy 18 with probe L1.84. *Hum Genet* 1986;74:346-352.
43. Bobrow MN, Harris TD, Shaughnessy KJ, Litt GJ. Catalyzed reporter deposition, a novel method of signal amplification. Application to immunoassays. *J Immunol Method* 1989;125:279-285.
44. Bobrow MN, Litt GJ, Shaughnessy KJ, Mayer PC, Conlon J. The use of catalyzed reporter deposition as a means of signal amplification in a variety of formats. *J Immunol Method* 1992;150:145-149.
45. Hopman AHN, Ramaekers FCS, Raap AK, Beck JLM, Devilee P, Van Der Ploeg M, Vooijs GP. In situ hybridization as a tool to study numerical chromosome aberrations in solid bladder cancer. *Histochem* 1989;89:307-316.

Chapter 3

Proliferation and apoptosis in primary B-cell non- Hodgkin's lymphoma

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Summary

To elucidate the role of cell turnover in primary gastric B-cell non-Hodgkin's lymphomas we studied tissue samples of 72 patients (26 small cell (25 MALT lymphomas), 46 large cell (31 MALT lymphomas)). Proliferative Indices (PI) and Apoptotic indices (AI) were measured by Mib-1 expression and terminal deoxynucleotidyl-transferase (TdT)-mediated nick end labelling, respectively. Furthermore, expression of the apoptosis related gene-products bcl-2 and p53 was studied.

Large cell lymphomas showed significantly higher PI (59.1% vs. 15.4%) and AI (3.2% vs 0.7%) than small cell lymphomas. Proliferative and Apoptotic Indices were positively correlated ($r = 0.371$, $p = 0.03$).

Expression of the bcl-2 protein was significantly higher in the small cell lymphomas. Furthermore, cases with a high bcl-2 expression showed both a significantly decreased PI ($p < 0.0001$) and AI ($p = 0.0096$) compared to bcl-2 negative cases. Expression of the mutant p53 protein was present in 8.0% of small cell and in 18.2% of large cell lymphomas. P53 positive cases showed significantly higher PI's, but showed no relation with AI.

These data suggest that impaired apoptosis by bcl-2 may be more prominent than proliferation in the genesis of small cell lymphomas whereas a high cell turnover characterises large cell primary gastric lymphomas.

Introduction

Primary gastric B-cell non-Hodgkin's lymphomas (NHL) are diagnosed with increasing frequency^{1,2}. It has been postulated that these lymphomas are a distinctive type of B-cell NHL, related to their clinical behaviour and pathological features³. Since their recognition as a separate group several lymphomas classifications have been adjusted to include these extranodal lymphomas, special classifications have been designed^{4,5}, and in the most recent lymphoma classification, proposed by the International Study Group on Lymphoma, small cell primary gastric lymphomas are classified as extranodal marginal zone NHLs⁶.

The balance between cell proliferation, cell differentiation and cell death determines the cell number in a population, and in this way the size of a tumour. To increase our understanding of the pathogenesis of neoplasms, therefore, regulation of cell proliferation as well as regulation of cell death are important features to investigate.

Like proliferation, apoptosis - the most important form of cell death- is an active process, requiring protein synthesis and endonucleolytic digestion of cellular DNA⁷. Several genes seem to be involved in the induction as well as the inhibition of apoptosis, such as the tumour suppressor gene p53, the c-myc gene, the bcl-2 gene and bcl-2 related genes^{8,9}. The functions of these genes can be changed at various levels. This may occur at the gene level by mutations or deletions like the p53 tumour suppressor gene, normally involved in the induction of apoptosis; at the RNA level by increased transcription, through, for example, juxtaposition of highly active genes and at the protein level¹⁰.

To elucidate the role of proliferation and apoptosis in primary gastric lymphomas, we analyzed 72 cases and studied these for the contribution of cell proliferation and apoptosis in the pathogenesis of these tumours. Proliferation was measured by staining with the Mib-1 antibody. For the detection of apoptosis we used the terminal deoxynucleotidyl transferase (TdT)-mediated digoxigenin-dUTP nick end labelling (TUNEL) assay, which is based on the detection of the 3'-OH nicks produced by apoptotic DNA fragmentation¹¹⁻¹³. Furthermore we studied the expression of the bcl-2 gene product¹⁴⁻¹⁷ and the p53 protein^{18,19} to get an impression of the regulatory processes involved in apoptosis, in this particular type of lymphoma.

Materials and Methods

Selection of cases

Cases were selected from the files of the pathology departments of nine hospitals in

Limburg and one hospital in Brabant (The Netherlands). All B-cell non-Hodgkin's lymphomas located in the stomach were selected and clinical data were retrieved to establish the diagnosis of primary gastric lymphoma, i.e. the stomach had to be the only or most massively affected organ. Furthermore, cases with a former nodal B-cell NHL were excluded. A further selection criterion was the availability of enough formalin-fixed, paraffin-embedded tissue blocks for proper diagnosis and analysis. At first, 83 primary gastric lymphomas were selected, but after review of the material, 11 cases were excluded, in 9 of which the material was insufficient; 1 case turned out to be a gastric carcinoma and in 1 case the diagnosis was a reactive process surrounding an ulcer. After these selections, 72 cases could be analyzed. In 18 of these only biopsy material was present; of the remaining 54 cases gastrectomy specimens were available.

Classification

Haematoxylin-eosin stained sections were reviewed by two pathologists (JWA, FJB) and the diagnosis of MALT lymphoma was based on the criteria described by Isaacson et al.^{3,20,21}, i.e. the presence of reactive B-cell follicles, surrounded by the tumour cell infiltrate consisting of centrocyte-like cells, selectively invading epithelial structures to form the characteristic lympho-epithelial lesions in addition to an immunohistochemically established B-cell phenotype of the tumour. When the tumour contained sheets of large blastic cells, cases were regarded as large cell (high grade) lymphoma. In these large cell cases a diagnosis of MALT lymphoma could be made only with confidence when the tumour contained a small cell (low grade) component displaying the typical features of MALT. When the characteristic features of MALT were absent, cases were classified as either small or large cell primary gastric B-cell lymphomas, and the MALT origin was considered uncertain, although in these cases the clinical diagnosis was primary gastric lymphoma. The inability to detect a small cell MALT component in large cell lymphomas was often due to the scarcity of tissue, especially in biopsy material. Table 3.1 shows the results of the classification.

Table 3.1 Classification of primary gastric lymphoma (n=72).

Small cell NHL	26
Malt features	25
Small cell primary gastric B-cell NHL (no MALT features present)	1
Large cell NHL	46
Large cell MALT component present	31
Large cell primary gastric B-cell NHL (MALT origin uncertain)	15

Immunohistochemistry for p53, bcl-2 and mib-1

Serial sections of 2 μm were cut from routinely processed formalin-fixed paraffin-embedded tissues and were mounted onto 3-Amino-Propyl-triethoxy-Silane (APS) coated slides. The sections were deparaffinized in xylene and rehydrated in graded ethanols. To undo the masking effect of formalin fixation, all sections were placed in a boiling citrate buffer (2.1 g citric acid in 1l of distilled H_2O ; pH to 6.0 with 5M NaOH) for 2 times 5 minutes in a microwave oven at 750 Watt^{22,23}. Outside the oven they were left to cool in the buffer for another hour. After washing in phosphate buffered saline (PBS) endogenous peroxidase was blocked with 0.3% H_2O_2 in methanol for 30 minutes. To block aspecific binding sites the slides were covered with 5% bovine serum albumin (BSA) in PBS. After washing in PBS the primary antibodies were applied: DO-7, a mouse monoclonal antibody that recognises an epitope in the N-terminus of the wild-type and mutant form of human p53 protein (DAKO, ITK diagnostics, Uithoorn, The Netherlands) diluted 1:500, incubation 2 hours at roomtemperature; Mib-1, a monoclonal mouse anti-human antibody which reacts to a nuclear antigen present in all phases of the cell cycle except for the G_0 phase (Dianova, Hamburg, Germany) diluted 1:100, incubation for 1 hour at roomtemperature and the monoclonal mouse anti-human bcl-2 oncoprotein, clone 124 (DAKO) diluted 1:100, incubation for 2 hours at roomtemperature. Negative controls were performed by omitting the primary antibody. As positive controls for bcl-2, mib-1 and p53, respectively, a follicular lymphoma, a reactive lymph node and a p53 positive breast-carcinoma were used. After washing in PBS, the primary antibody was detected by incubation with biotinylated anti-mouse antibody (Vectastain Elite Kit, Vector, Burlingame, CA, USA) for 30 minutes at room temperature. After washing peroxidized avidin-biotin complex (Vectastain Elite Kit) was applied for 30 minutes at room temperature. Visualisation was achieved with 3.3' diaminobenzidine-tetrahydrochloride (DAB) in a Tris-HCl buffer. The slides were counterstained lightly with haematoxylin. After dehydration in graded alcohols and xylene they were mounted with entellan.

Terminal deoxynucleotidyl transferase (TdT)-mediated digoxigenin-dUTP nick end labelling (TUNEL assay)

Sections of 2 μm were deparaffinized and rehydrated. Endogenous peroxidase was blocked as described above. Sections were pretreated with 20 $\mu\text{g}/\text{mL}$ of protein digesting enzyme (ONCOR, Sanbio, Uden, The Netherlands) for 15 minutes at room temperature. After washing in 4 changes of aquadest, equilibration buffer (Apoptag Kit, Oncor, Gaithersburg, Maryland, USA) was applied for 10 minutes at room temperature, followed by the application of 13 μl working strength TdT enzyme (Apoptag Kit) per cm^2 tissue section for one hour at 37° Celsius in a humidified chamber and covered with a coverslip. The reaction was terminated in

preheated Stop/Wash buffer (Apoptag Kit) for 30 minutes at 37° Celsius. After washing in PBS, the sections were covered with anti-digoxigenin-peroxidase (Apoptag Kit) for 30 minutes at roomtemperature and after washing visualisation was performed by DAB, the colour reaction was followed by light-microscopy and terminated when adequate staining was achieved. Sections were lightly counterstained with haematoxylin and after dehydration were mounted with entellan. As a positive control a routinely formalin-fixed and paraffin-embedded tissue block of rat thymus was processed as described above, and as a negative control, the TdT enzyme in the working strength TdT-enzyme was replaced by aquadest.

Evaluation

In general, the p53 and mib-1 positive nuclei were counted in a total of at least 500 neoplastic cells with the aid of a counting grid at a magnification of 400 times. At least three high power fields were examined. From these observations the mean relative frequency (%) was calculated. More specifically, in the case of p53 staining, only tumours that contained more than 30% positive nuclei were regarded as bearing p53 mutations. Cases expressing a lower percentage were considered to possibly express wild type p53²⁴.

Regarding bcl-2, three staining patterns were distinguished: high expression of bcl-2 (almost all tumour cells expressing bcl-2), intermediate expression (bcl-2 positive and negative tumour cells admixed in the same area, but less than 60% positive cells) and negative expression (virtually all tumour cells negative, but bcl-2 positive small reactive lymphocytes could be present)²⁵. In the analysis of the TUNEL assay, the apoptotic indices (AI) were determined by the ratio of TUNEL-positive neoplastic cells/total number of neoplastic cells, and were calculated in at least five microscopical fields to a total of at least 1000 tumour cells (magnification 400x).

Statistical analysis

For statistical evaluation of the results SPSS 6.1 was applied using the Mann-Whitney U test, the Kruskal-Wallis 1-way Anova and the Spearman correlation coefficient for non-parametric variables. A p-value less than 0.05 was considered significant.

Results

Results in MALT cases versus uncertain MALT cases

We performed all analyses both for all cases as well as for the MALT cases only. There were no gross differences in outcome, therefore, we will discuss all primary gastric lymphoma cases together.

Proliferation-Index (PI) in primary gastric lymphoma

In 70 cases strong nuclear immunoreactivity was seen for the proliferation marker Mib-1. The mean PI was 43.51%. In small cell lymphomas we found a mean PI of 15.40% and in large cell lymphomas a mean PI of 59.13%. The difference between these two histologic classes was significant (Mann-Whitney U Test, $p < 0.0001$).

Bcl-2 expression in primary gastric lymphoma

Sixty-nine cases could be evaluated for bcl-2 staining. The bcl-2 protein was expressed in the tumour cells of 38 (55.1%) cases, of which 14 (20.3% of total cases) showed an intermediate staining pattern, with positive and negative cells admixed in the same area, and the remaining 24 (34.8% of total cases) demonstrated a high bcl-2 expression, with staining of virtually all tumour cells. In 31 cases (44.9%) the tumour cells were predominantly negative. Most of the small cell MALT lymphomas showed high bcl-2 expression (18 out of 24 cases, 75.0%), whereas a minority displayed an intermediate staining pattern or negative bcl-2 expression (both categories 12.5%). Small cell MALT lymphomas with a high bcl-2 expression showed a typical staining pattern in the follicles, with a negative follicle centre and a surrounding bcl-2 positive mantle zone and marginal zone. Twenty-eight out of 45 large cell lymphomas (62.3%) showed bcl-2 negative tumour cells and fewer cases had an intermediate staining pattern (24.4%) or a high bcl-2 expression (13.3%). Figure 3.1 (see appendix) shows the results of bcl-2 and mib-1 staining in small and large cell primary gastric lymphoma.

p53 expression in primary gastric lymphoma

A total of 10 out of the 69 evaluated cases (14.5%) showed p53 overexpression, and all of these cases overexpressed p53 in almost all tumour cells. The majority of these positive cases were of large cell histology ($n=8$) whereas only 2 small cell lymphomas were p53 positive. In total 18.2% of large cell lymphomas were p53 positive and 8.0% of small cell lymphomas were positive. This difference between small and large cell lymphomas was not significant ($p=0.248$, Chi-Square test).

Apoptotic Index (AI) in primary gastric lymphoma: results of the TUNEL assay

To optimize TUNEL immunoreactivity, we first varied the concentration and incubation time of protein digestion (protein digesting enzyme, ONCOR, Sanbio). Furthermore, because of the report of an enhancement of immunoreactivity after microwave irradiation in citrate buffer²⁶, we tested several durations of microwave heating. We did not find an improvement in immunoreactivity, but we noticed a

decline in staining intensity and sensitivity after long exposure times. After these tests, we used the protocol described.

All tumours showed immunoreactivity, and apoptotic cells and apoptotic bodies were seen in between viable tumour cells. A variable proportion of stained cells showed morphological criteria of apoptosis: peripherally beaded or shrunken nodular condensed chromatin. Some nuclei were strongly labelled without obvious morphological features of apoptosis, which might represent an early stage of apoptosis. Some phagocytosed apoptotic bodies could be detected in macrophages, which then also showed some cytoplasmic staining. The latter cases were not counted in the AI. In a few cases, in which the AI was very high, some of the morphologically apoptotic cells did not stain positive. These non-stained cells were also not counted in the AI. Figures 1E and 1F (see appendix) show TUNEL staining in small and large cell lymphomas.

In general, we saw low percentages of TUNEL positive cells with a mean of 2.26% positive tumour cells. In large cell lymphomas this percentage was higher than in small cell lymphomas (3.23% versus 0.71%). This difference between small and large cell lymphoma was significant (Mann-Whitney U Test, $p=0.0020$)

Relation between PI and AI

To investigate if tumours with higher proliferation indices also had higher apoptotic indices and in this way a higher cell turnover rate we evaluated the correlation between these two variables (Figure 3.2). The Spearman correlation coefficient for non-parametric variables showed a loose ($r=0.3713$) correlation. This was, however, significant ($p=0.03$).

Relation between bcl-2 and PI (Mib1) and AI (TUNEL)

Subsequently, we analyzed the relationship between Proliferative and Apoptotic Indices and bcl-2 expression. Cases with a negative bcl-2 expression were more likely to have a high PI, whereas cases with a high expression of bcl-2 were more likely to have a low PI (Figure 3.3). As can be noticed from this figure, the Proliferation Index decreases with higher amounts of bcl-2 positive cells. This was significant for both small and large cell cases ($P<0.0001$, Kruskal-Wallis 1-Way Anova). In figure 3.4 the relation between bcl-2 and the Apoptotic Index is shown. Cases with high bcl-2 expression were more likely to show a lower Apoptotic Index. We found a significantly lower AI in cases with a high bcl-2 expression when compared either to cases with an intermediate bcl-2 expression ($p<0.05$) or to bcl-2 negative cases ($p<0.05$, Mann-Whitney U-test). The difference in AI between bcl-2 negative and bcl-2 intermediate cases was not significant.

Figure 3.2 Association between Proliferation Index (PI) and Apoptotic Index (AI) in small (●) and large (○) cell primary gastric lymphoma. Spearman correlation coefficient: 0.3713, $p=0.03$.

Figure 3.3 Significant decrease in Proliferation Index (PI) with increasing bcl-2 expression in small (●) ($n=24$) and large (○) ($n=45$) cell primary gastric lymphoma.

Figure 3.4 Relation between Apoptotic Index (AI) and bcl-2 expression pattern in primary gastric lymphoma ($n=54$, ●=small cell, ○large cell). Apoptotic Indices were lower in cases with a high bcl-2 expression compared to bcl-2 intermediate and negative cases ($p<0.05$).

p53 overexpression in relation to bcl-2 staining, PI and AI

Only 10 cases were positive for p53, 5 of which were bcl-2 negative, 4 showed intermediate bcl-2 staining and 1 demonstrated high bcl-2 expression. There was no significant relationship between bcl-2 and p53 expression (Chi-square statistics, $p=0.09580$). Relating p53 expression to Proliferation Index, we found significantly higher Proliferative Indices in p53 positive cases compared to p53 negative cases ($p<0.05$, Mann-Whitney U-test). In the case of Apoptotic Indices, however, we did not find a significant relationship (Mann-Whitney U-test, $p=0.909$).

Discussion

In order to analyze the relative distribution of cell proliferation and prolonged survival in the pathogenesis of primary gastric B-cell non-Hodgkin's lymphoma, we studied 72 cases to determine the Proliferative Index (PI) and the Apoptotic Index (AI) respectively by Mib-1 and TUNEL reactivity. Furthermore, we choose to study two gene-products involved either in the induction (wild-type p53) or in the inhibition of apoptosis (bcl-2). There appeared to be a significant correlation of both PI as well as AI with histology: small cell lymphomas showed significantly lower AI and PI than large cell lymphomas. This is in accordance with earlier reports in gastro-intestinal non-Hodgkin's lymphomas^{27,28}. When analysing the correlation between these two components that determine cell turnover²⁹, we found that higher PI's are significantly correlated with higher AI's, which has been shown before in nodal lymphomas³⁰. Combining these two results, it appears that small cell gastric lymphomas show a low proliferation index, in combination with a low apoptotic index, i.e., a low cell turnover rate. In contrast large cell lymphomas showed a high proliferation index as well a high apoptotic index.

The role of the bcl-2 protein, which is one of the most important gene-products involved in apoptosis, may be of relevance. In follicular lymphoma bcl-2 is overexpressed as a result of a t(14;18) translocation. In MALT lymphoma, follicles are reactive and indeed, in all of our small cell cases showing MALT features the follicles were bcl-2 negative, whereas the surrounding mantle and marginal zones were bcl-2 positive. This bcl-2 expression in small cell primary gastric lymphomas as observed in our study corresponds to the idea that bcl-2 inhibits apoptosis, as we saw lower AI in our small cell cases. In contrast, the majority of large cell lymphomas was bcl-2 negative, and apoptosis rates were considerably higher than in small cell lymphomas. This could be in accordance with a lack of inhibition of apoptosis by bcl-2 in large cell lymphomas. These results are in agreement with findings by others, who also found high bcl-2 expression in low grade MALT lymphomas and very rare bcl-2 expression in large cell MALT lymphomas^{31,32}.

Bcl-2 gene rearrangements, however, were not detected in MALT lymphomas³³. Apparently, an alternative, yet unknown, mechanism other than bcl-2 rearrangement is responsible for the (over)expression of bcl-2 in small cell lymphomas³⁴. Regarding p53 immunoreactivity, we saw that a very low percentage of tumours showed p53-mutations, which is in accordance with previous reports^{35,36}. The fact that 8 out of 10 p53 positive cases were large cell lymphomas may suggest that there could be a role for p53 gene mutations in the progression from small to large cell lymphomas. The p53 gene product is involved in the DNA repair machinery. Mutation of the p53 gene leads to a condition in which apoptosis is no longer induced by p53 in response to DNA damage. In this respect we might expect that tumours bearing mutated p53 protein should have lower apoptotic indices, but we did not find such a relationship in our material. This could of course be due to the low percentage of p53-positive cases, indicating that p53-mutations are not of major importance in primary gastric lymphomas.

Our study underscores the importance of apoptosis in relation to proliferation in gastric lymphomas, and probably in oncogenesis in general³⁷. New directions of therapy, therefore, should focus more on influencing this process.

References

1. Hayes J, Dunn E. Has the incidence of primary gastric lymphoma increased ? *Cancer* 1989;63:2073-2076.
2. Severson RK, Davis S. Increasing incidence of primary gastric lymphoma. *Cancer* 1990;6:183-187.
3. Isaacson PG, Wright DH. Malignant lymphoma of mucosa-associated tissue. A distinctive type of B-cell lymphoma. *Cancer* 1983;52:1410-1416.
4. Rohatiner A, d'Amore F, Coiffier B, Crowther D, Gospodarowicz M, Isaacson P, Lister TA, Norton A, Salem P, Shipp M, Somers R. Report on a workshop convened to discuss the pathological and staging classifications of gastro-intestinal tract lymphoma. *Ann Oncol* 1994;5:397-400.
5. Isaacson PG, Spencer J, Wright DH. Classifying primary gut lymphomas. *Lancet* 1988;2:1148-1149.
6. Harris NL, Jaffe ES, Stein H, Banks PM, Chan JKC, Cleary ML, Delsol G, De Wolf-Peeters C, Falini B, Gatter KC, Grogan TM, Isaacson PG, Knowles DM, Muller-Hermelink HK, Pileri SA, Piris MA, Ralfkiaer E, Warnke RA. A revised European-American classification of lymphoid neoplasms: a proposal from the International Lymphoma Study Group. *Blood* 1994;84:1361-1392.
7. Wyllie AH, Kerr JRR, Currie AR. Cell death: the significance of apoptosis. *Int Rev Cytol* 1980;68:251.
8. Hale AJ, Smith CA, Sutherland LC, Stoneman VEA, Longthorne VL, Culhane AC, Williams GT. Apoptosis: molecular regulation of cell death. *Eur J Biochem* 1996;236:1-26.
9. Hoffman B, Liebermann DA. Molecular controls of apoptosis: differentiation/growth arrest primary response genes, proto-oncogenes, and tumor suppressor genes as positive & negative modulators. *Oncogene* 1994;9:1807-1812.
10. Tsujimoto Y, Ikegaki N, Croce CM. Characterization of the protein product of bcl-2, the gene involved in human follicular lymphoma. *Oncogene* 1987;2:3-7.
11. Gavrieli Y, Sherman Y, Ben-Sasson SA. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J Cell Biol* 1992;119:493-501.
12. Wijsman JH, Jonker RR, Keijzer R, Van De Velde CJH, Cornelisse CJ, Van Dierendonck JH. A new method to detect apoptosis in paraffin sections: in situ end-labeling of fragmented DNA. *J Histochem Cytochem* 1993;41:7-12.
13. Gorzycza W, Gong J, Darzynkiewicz Z. Detection of DNA strand breaks in individual apoptotic cells by the in situ terminal deoxynucleotidyl transferase and nick translation assays. *Cancer Res* 1993;53:1945-1951.
14. Hockenbery D, Nunez G, Miliman C, Schreiber RD, Korsmeyer SJ. Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature* 1990;348:334-346.
15. Korsmeyer SJ. Bcl-2 initiates a new category of oncogenes: regulators of cell death. *Blood* 1992;80:879-866.

16. Reed JC. Bcl-2 and the regulation of programmed cell death. *J Cell Biol* 1994;124:1-6.
17. Farrow SN, Brown, R. New members of the bcl-2 family and their protein partners. *Curr Opin Gen Dev* 1996;6:45-49.
18. Bates S, Vousden KH. p53 in signaling checkpoint arrest or apoptosis. *Curr Opin Gen Dev* 1996;6:12-19.
19. Agarwal ML, Agarwal A, Taylor WR, Stark GR. p53 controls both the G2/M and the G1 cell cycle checkpoints and mediates reversible growth arrest in human fibroblasts. *Proc Natl Acad Sci USA* 1995;92:8493-8497.
20. Isaacson PG, Spencer J. Malignant lymphoma of mucosa-associated lymphoid tissue. *Histopathology* 1987;11:445-462.
21. Isaacson PG. Lymphomas of mucosa-associated lymphoid tissue (MALT). *Histopathology* 1990;16:617-619.
22. Cattoretti G, Pileri S, Parravicini C, Beckers MHG, Poggi S, Bifulco C, Key G, D'Amato L, Sabattini E, Feudale E, Reynolds F, Gerdes J. Antigen unmasking on formalin fixed, paraffin-embedded tissue sections. *J Pathol* 1993;171:83-98.
23. Shi S, Key ME, Kalra KL. Antigen Retrieval in formalin-fixed, paraffin-embedded tissues: an enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. *J Histochem Cytochem* 1991;9:741-748.
24. Hall PA, Lane DP. P53 in tumour pathology: Can we trust immunohistochemistry? - Revisited ! *J Pathol* 1994;172:1-4.
25. Hermine O, Haioun C, Lepage E, D'Agay MF, Briere J, Lavignac C, Fillet G, Salles G, Marolleau JP, Diebold J, Reyes F, Gaulard P. Prognostic significance of bcl-2 protein expression in aggressive non-Hodgkin's lymphoma. *Blood* 1996;87:265-272.
26. Sträter J, Günthert AR, Brüderlein S, Möller P. Microwave irradiation of paraffin-embedded tissue sensitizes the TUNEL method for in situ detection of apoptotic cells. *Histochemistry* 1995;103:157-160.
27. Mikou P, Kanavaros P, Aninos D, Tzardi M, Karameris A, Gorgoulis B, Papadopoulos N, Lavergne A, Galian A. Nuclear organizer regions (NORs) staining and proliferating cell nuclear antigen (PCNA) immunostaining in mucosa-associated lymphoid tissue (MALT) gastric lymphomas. *Pathol Res Pract* 1993;189:1004-1009.
28. Woods AL, Hall PA, Shepherd NA, Hanby AM, Waseem NH, Lane DP, Levison DA. The assessment of proliferating cell nuclear antigen (PCNA) immunostaining in primary gastrointestinal lymphomas and its relationship to histological grade, S+G₂+M phase fraction (flow cytometric analysis) and prognosis. *Histopathology* 1991;19:21-27.
29. Spina D, Leoncini L, Del Vecchio M.T. Megha T, Minacci C, Poggi SA, Pileri SA, Tosi P, Kraft R, Laissue JA, Cottier H. Low versus high cell turnover in diffusely growing non-Hodgkin's lymphomas. *J Pathol* 1995;177:335-341.
30. Leoncini L, Del Vecchio MT, Megha T, Barbini P, Galieni P, Pileri S, Sabattini E, Gherlinzoni F, Tosi P, Kraft R, Cottier H. Correlations between apoptotic and proliferative indices in malignant non-Hodgkin's lymphomas. *Am J Pathol* 1993;142:755-763.

31. Ashton-Key M, Biddolph SC, Stein H, Gatter KC, Mason DY. Heterogeneity of bcl-2 expression in MALT lymphoma. *Histopathology* 1995;26:75-78.
32. Navratil E, Gaulard P, Kanavaros P, et al. Expression of the bcl-2 protein in B cell lymphomas arising from mucosa associated lymphoid tissue. *J Clin Pathol* 1995;48:18-21.
33. Pan L, Diss TC, Cunningham D, Isaacson PG. The bcl-2 gene in primary B cell lymphoma of mucosa-associated lymphoid tissue (MALT). *Am J Pathol* 1989;135:7-11.
34. Pezzella F, Tse AGD, Cordell JL, Pulford KAF, Gatter KC, Mason DY. Expression of the bcl-2 oncogene protein is not specific for the 14;18 translocation. *Am J Pathol* 1990;37:225-232.
35. Du M, Peng H, Singh N, Isaacson PG, Pan L. The accumulation of p53 abnormalities is associated with progression of mucosa-associated lymphoid tissue lymphoma. *Blood* 1995;86:4587-4593.
36. Nakamura S, Akazawa K, Kinukawa N, Takashi Y, Tsuneyoshi M. Inverse correlation between the expression of bcl-2 and p53 proteins in primary gastric lymphoma. *Hum Pathol* 1996;27:225-233.
37. Hsu B, Marin MC, McDonnell TJ. Cell death regulation during multistep lymphomagenesis. *Cancer Letters* 1995;94:17-23.

Chapter 4

Specific detection of *Helicobacter pylori* and non- *Helicobacter pylori* flora in small- and large-cell primary gastric B-cell non-Hodgkin lymphoma

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Summary

Background

Primary gastric non-Hodgkin's lymphomas possibly develop in response to local infection by *Helicobacter pylori* (*H.pylori*). We investigated the presence of *H.pylori* and non-*H.pylori* flora histologically in small and large cell primary gastric lymphoma using a specific staining method.

Materials and methods

Specimens of 52 cases of primary gastric lymphoma (17 small cell, 35 large cell) were stained with modified Giemsa (MG) and immunohistochemically using a polyclonal antibody against *H.pylori* (IHC).

Results

Thirty-two cases (61.5%) (Small cell 76% versus large cell 53%, $p > 0.05$) showed immunoreactivity for *H.pylori* in the mucosa surrounding the tumour. Remarkable was the localisation of *H.pylori* in the neck of the gastric glands in 3 cases. Non-*H.pylori* flora was seen in 35 cases (76.3%) (small cell 53% versus large cell 74%, $p > 0.05$). In 20 cases this non-*H.pylori* flora was mixed with *H.pylori*. Five cases showed no bacterial flora at all.

Conclusions

1. Using immunohistochemistry the prevalence of gastric lymphoma cases with *H.pylori* (61.5%) approximates that of *H.pylori* in the normal population.
2. No statistical difference was found between the occurrence of *H.pylori* and of non-*H.pylori* bacterial flora in small versus large cell lymphoma.
3. Our results suggest that *H.pylori* may not be the only etiologic factor in primary gastric lymphoma.

Introduction

Studies investigating the pathogenesis of gastric carcinomas have found an important role in the etiology of this condition for *Helicobacter pylori* (*H.pylori*)¹. This bacterium may also be involved in the etiology of primary gastric lymphoma, which is the commonest extranodal non-Hodgkin's lymphoma. Although it accounts for only a minority of gastric neoplasms, its incidence is increasing². *H.pylori* is considered to cause a follicular gastritis in the stomach and to be responsible for the development of lymphoid tissue in the stomach, where normally no organized lymphoid tissue is present. It is hypothesized that malignant lymphomas could evolve from this acquired mucosa-associated lymphoid tissues (MALT). The observation that eradication therapy for *H.pylori* in some cases can lead to regression of primary low grade gastric lymphomas may support this hypothesis³⁻⁵.

Studies investigating the presence of *H.pylori* in gastric mucosa surrounding MALT lymphoma using non-specific staining methods have found the bacterium in 41% up to 92% of cases. These differences prompted us to study *H.pylori* with a more sensitive and specific immunohistochemical staining method^{6,7}. Despite the fact that most gastric lymphomas are large cell lymphomas, most studies on the occurrence of *H.pylori* have focused on small cell gastric MALT lymphoma. It may, however, also be interesting to look for the presence of *H.pylori* in large cell lymphoma. Finally, by using this specific staining technique for the detection of *H.pylori* in addition to a non-specific staining method (modified Giemsa) we were also able to evaluate the presence of other bacteria present in primary gastric lymphomas and therefore could evaluate a possible role for non-*H.pylori* flora in the pathogenesis of gastric lymphomas.

Materials and methods

Gastric lymphoma cases were selected from the files of the pathology departments of ten hospitals in the provinces of Limburg and Brabant, the Netherlands. Clinical data were retrieved to establish that the lymphoma was primary gastric, i.e. the stomach being the only or most massively affected organ, and no former nodal disease was present. All formalin-fixed, paraffin-embedded tissue blocks from either biopsies or gastrectomies were collected. Only cases in which not only tumour tissue but also normal gastric mucosa was present were included in this study, for two reasons: firstly, a more detailed classification of MALT (i.e. more chance to detect a low grade MALT component in high grade lymphoma), and secondly because of the possibility that the occurrence of *H.pylori* could be

confined to 'normal' mucosa. Haematoxylin-eosin stained sections were reviewed by two pathologists (JWA, FB) and diagnosis of MALT lymphoma was based on the criteria described by Isaacson et al.⁸⁻¹⁰, i.e. presence of reactive B-cell follicles, surrounded by the tumour cell infiltrate consisting of centrocyte-like cells, selectively invading epithelial structures to form characteristic lympho-epithelial lesions. When the tumour contained fields of large blastic cells, cases were regarded as large cell (high grade) lymphoma. Unless these large cell tumours contained a low grade component displaying the typical features of MALT, no diagnosis of large cell MALT lymphoma could be made and they were diagnosed as large B-cell lymphoma. Using these criteria fifty-two cases could be selected (31♂, 21♀, mean age at diagnosis: 62 years). At least two tissue blocks were present for each case, with a maximum of 14 blocks. Based on the classification, 17 patients had small cell lymphoma, in all of which MALT features could be detected, 26 had a large cell lymphoma of MALT with a small cell MALT component and 9 patients had a large B-cell lymphoma of the stomach without a small cell MALT component.

Diagnosis of *H.pylori* was made by assessment of modified Giemsa (MG) and immunohistochemistry (IHC) stained serial sections of 4 µm. MG staining was performed as described by Gray¹¹. For the immunohistochemical staining a purified *H.pylori* antiserum was used (DAKO B471, ITK Diagnostics BV, Uithoorn, The Netherlands) in a 1:500 dilution. Staining was performed as described by Loffeld et al.⁶ without pepsin treatment and with biotin-conjugated swine-anti-rabbit antibodies (DAKO E431) as a second layer, followed by StreptABC-HRP (DAKO K377). All slides were examined at a magnification of 400 times, by two observers. In the MG stain, *H.pylori* was diagnosed by its characteristic S-shaped appearance and the typical localisation in or on the gastric mucus layer. *H.pylori* positivity was defined as the presence of immunoreactivity. By joint evaluation of both IHC and MG stains, non-*H.pylori* flora was assumed when bacterial flora observed in MG were negative in IHC staining. These bacteria were not further characterized in this study. The presence of *H.pylori* was scored on a semi-quantitative scale: grade 0: no bacteria seen, grade 1: sporadic bacteria observed, grade 2: bacteria present in almost all high power fields, 3: bacteria abundantly found in all high power fields, also lying in clusters. The Chi-square test (and in case of small samples Fisher's Exact Test) was applied for statistical analysis. A p-value of less than 0.05 was considered to be significant.

Results

Detection of *H.pylori* by IHC revealed a positive reaction in 32 cases (61.5%). Figure 4.1 (see appendix) shows an example of both a MG and an IHC stain in the

same lymphoma, both showing *H.pylori* flora. In all cases, *H.pylori* was detected in the mucosa surrounding the tumour. It was never detected inside the lymphoma tissue. The presence of *H.pylori* varied from grade 1 to grade 3. In twelve cases grade 1 was the highest grade, in six cases grade 2 and in ten cases we noticed grade 3. Using the MG stain *H.pylori* was suspected in four additional cases, which were negative on IHC. The MG stained sections were also examined for the presence of other bacteria, apart from the presence of *H.pylori*. In this way we saw non-*H.pylori* flora in 35 cases (67.3%) (20 in *H.pylori* positive cases and 15 in *H.pylori* negative cases). Non-*H.pylori* flora was mainly seen in strains of mucus overlying the mucosa, and also in the mucosal crypts. In cases with mixed flora, i.e. *H.pylori* as well as non-*H.pylori* flora, both flora could sometimes be detected in the same crypts. In 5 cases neither *H.pylori* nor non-*H.pylori* flora were found. *H.pylori* was detected in more small cell cases (76.5%) than large cell cases (54.3%), although this difference was not significant. In large cell lymphomas without a small cell MALT component, however, a lower percentage (33.3%) showed immunohistochemical presence of *H.pylori*. The presence of non-*H.pylori* flora was higher in the large cell lymphomas (74.3%) compared to the small cell cases (52.9%). However, the difference between the histological classes was not significant (Table 4.1). In figure 4.2 (see appendix), a case of primary gastric lymphoma is shown in which *H.pylori* was present deep in the gastric pits, at the level of the neck of the glands. We noticed this phenomenon in 3 cases (2 small cell MALT cases, 1 large cell lymphoma of MALT origin).

Discussion

Gastric MALT lymphomas account for the majority of extranodal MALT lymphoma, which is paradoxical, since the normal gastric mucosa is devoid of any organized lymphoid tissue. Therefore, the hypothesis was formulated that there should be a stimulus for the development of MALT in the stomach. Since the description of *H.pylori* by Warren et al.¹², research on *H.pylori* has extended exponentially^{13,14}, and its role in the pathogenesis of gastritis, peptic ulcer and gastric cancer^{1,15,16} has been investigated. In the case of gastric lymphoma, the development of lymphoid tissue is important in its pathogenesis and studies have shown that stomachs infected by *H.pylori* show acquired lymphoid follicles and aggregates^{17,18}. This lymphoid tissue may be a precursor of gastric lymphoma. Several groups have investigated the occurrence of *H.pylori* in gastric lymphomas, either by histological detection or by serology. The reported frequency of *H.pylori* in primary gastric lymphoma varies considerably, from 41% in a study of Calvert et al.¹⁹, 50% in a study of Karat et al.²⁰, 59% (Miettinen et al.²¹), 80% (Muller et al.²²) to a maximum of 92% in a study of Wotherspoon et al.²³ when detected

histologically. In spite of a common approach, using a routine haematoxylin-eosin staining, and only staining cases which were negative and/or in which additional material was present with the MG stain, considerably varying percentages of *H. pylori* were reported.

Table 4.1 *Helicobacter pylori* and non-*Helicobacter pylori* flora in low grade MALT lymphomas, high grade lymphomas with a low grade MALT component and high grade gastric lymphomas without a low grade MALT component¹.

Histology Staining results	<i>H. pylori</i> (IMM+/MG-)	Non- <i>H. pylori</i> flora only (IMM-/MG+)	Both <i>H. pylori</i> as well as non- <i>H. pylori</i> flora (mixed) (IMM+/MG+)	No flora (IMM-/MG-)
Small cell MALT (n=17)	13 (76.5%) ^a	3 (17.6%)	6 (35.3%)	1 (5.9%)
Large cell (n=35)	19 (54.3%)	12 (34.3%)	14 (40.0%)	4 (11.4%)
- with a small cell MALT component (n=26)	16 (61.5%)	7 (26.9%)	12 (46.2%)	3 (11.5%)
- without proven MALT component (n=9)	3 (33.3%) ^a	5 (55.6%)	2 (22.2%)	1 (11.1%)
Total (n=52)	32 (61.5%)	15 (28.8%)	20 (38.5%)	5 (9.6%)

¹ χ^2 -statistics were used to determine differences in the distribution of *H. pylori*, non-*H. pylori* flora only, mixed flora and no flora, between small cell MALT vs large cell cases, small cell MALT vs large cell with a small cell MALT component, small cell MALT vs large cell without certain MALT component and large cell MALT vs large cell without a certain MALT component. ^a Significant difference in distribution of *H. pylori* (p=0.042, Fisher's Exact Test)

Using IHC for the detection of *H. pylori* in all cases allowing a more specific identification was an important feature of our study. We could detect *H. pylori* by immunohistochemistry in 32 out of 52 primary gastric lymphoma cases (61.5%). Including the cases which were (false)-positive in MG and negative in IHC this percentage was 69.2%. Besides the choice of a more or less specific and/or sensitive staining method additional explanations like atrophic changes in the body mucosa^{21,24}, differences in fixation of the specimens²⁵, availability of gastrectomy specimens²⁶, presence of non-tumorous mucosa, previous eradication therapy of *H. pylori* (which plays no important role in the present series, because all patients were included before 1992), extent of intestinal metaplasia²⁷ and experience of the observers to identify *H. pylori* can explain variations in detected frequencies. The percentage of *H. pylori* found in this study is close to the mean percentage found in the asymptomatic European population of this age (62,4%)²⁸. Although no

serological studies of *H.pylori* in the asymptomatic population of the same region are available, Loffeld et al. found a percentage of about 70% in dyspeptic patients²⁹. In earlier studies, which reported higher percentages of *H.pylori*, an association with primary gastric lymphoma was suggested because of these higher percentages. Our findings of more commonly observed percentages makes it plausible that *H.pylori* is not the sole causative factor in gastric lymphoma but that additional etiologic factors are necessary for the development of MALT and gastric lymphoma.

Comparing the findings in small cell primary gastric MALT lymphoma and the total of large cell gastric lymphomas (i.e. with or without a small cell MALT component), no significant difference in the occurrence of *H.pylori* was found. Assuming that *H.pylori* provides an antigenic drive for the proliferation of gastric lymphoma^{30,31} this suggests that large cell lymphomas could still be dependent on this drive. The finding of lower frequencies of *H.pylori* in large cell lymphomas without a small cell MALT component compared to both small MALT (significant) as well as large cell lymphomas with a small cell MALT component (not significant) might suggest that *H.pylori* plays a minor role in lymphomas without a MALT origin. Moreover, these cases showed the most intense non-*H.pylori* flora, which might suggest a more important role of non-*H.pylori* flora in these lymphomas. However, it should be borne in mind that in these cases little mucosal tissue was present, which interfered both with the detection of *H.pylori* as well as with the diagnosis of MALT. Furthermore, the high intensities of non-*H.pylori* flora in this study may be the result of local changes induced by the tumour, like an increased pH, causing a more intestinal-like bacterial flora.

The observation of *H.pylori* in three cases in the neck of the gastric glands without their presence in the superficial mucosa is interesting. To our knowledge, such a localisation of *H.pylori* has never been described before. We hypothesize that this unusual localisation could be a result of a non-active infection with *H.pylori*, retracted in the deep layers of the mucosa but not completely removed by the immune system. This equilibrium and its disturbances may play a role in the development of organized lymphoid tissue in the stomach.

In conclusion, our results suggest that the occurrence of *H.pylori* in primary gastric B-cell non-Hodgkin's lymphoma is not different from its occurrence in the normal (European) population. Furthermore, we found no significant difference between small cell MALT lymphoma and large cell lymphoma with a proven MALT component and we found a high frequency of other bacteria. Although there is evidence for a role of *H.pylori* in the etiology of primary gastric lymphoma, the abundance of non-*H.pylori* flora suggests that *H.pylori* might not be the only etiologic factor in the development of gastritis, MALT and primary gastric lymphoma. For this reason, and because the presence of *H.pylori* in some cases is difficult to establish using non-specific staining methods, its histological presence is

best examined by IHC, for example when considering eradication therapy for gastric lymphoma. Furthermore, one should realise that small-spectrum antibiotic therapy directed to *H.pylori* might not eradicate other bacteria that could be a persisting drive for the development of MALT or gastric lymphoma.

References

1. Nomura A, Stemmermann GN. *Helicobacter pylori* and gastric cancer. J Gastroen Hepatol 1993;8:294-303.
2. Severson RK, Davis S. Increasing incidence of primary gastric lymphoma. Cancer 1990;66:1283-1287.
3. Weber DM, Dimopoulos MA, Anandu DP, Pugh WC, Steinbach G. Regression of gastric lymphoma of mucosa-associated lymphoid tissue with antibiotic therapy for *Helicobacter pylori*. Gastroenterology 1994;107:1835-1838.
4. Roggero E, Zucca E, Pinotti G, Pascarella A, Capella C, Sario A, Pedrinis E, Paterlini A, Venco A, Cavalli F. Eradication of *helicobacter pylori* infection in primary low-grade gastric lymphoma of mucosa-associated lymphoid tissue. Ann Intern Med 1995;122:767-769.
5. Wotherspoon AC, Doglioni C, Diss TC, Pan L, Moschini A, De Boni M, Isaacson PG. Regression of primary low-grade B-cell gastric lymphoma of mucosa-associated lymphoid tissue type after eradication of *Helicobacter pylori*. Lancet 1993;342:575-577.
6. Loffeld RJLF, Stobberingh E, Flendrig JA, Arends JW. *Helicobacter pylori* in gastric biopsy specimens. Comparison of culture, modified Giemsa Stain, and immunohistochemistry. A Retrospective study. J Pathol 1991;165:69-73.
7. Andersen LP, Holck S, Poulsen CO. *Campylobacter pylori* detected by indirect immunohistochemical technique. APMIS 1988;96:559-564.
8. Isaacson P, Wright DH. Malignant lymphoma of mucosa-associated lymphoid tissue. Cancer 1983;52:1410-1416.
9. Isaacson PG, Spencer J. Malignant lymphoma of mucosa-associated lymphoid tissue. Histopathology 1987;11:445-462.
10. Isaacson PG. Lymphomas of mucosa-associated lymphoid tissue (MALT). Histopathology 1990;16:617-619.
11. Gray SF, Wyatt JJ, Rathbone BJ. Simplified techniques for identifying *Campylobacter pyloridis*. J Clin Pathol 1986;39:1280.
12. Warren JR. Unidentified curved bacilli on gastric epithelium in active chronic gastritis. Lancet 1983;1:1273.
13. Genta RM, Graham DY. *Helicobacter pylori*: the new bug on the (paraffin) block. Virchows Arch 1994;425:339-347.
14. Talley NJ. *Helicobacter pylori*: More than 10 years on and still catchy. J Gastroen Hepatol 1995;10:465-468.
15. Rautelin H, Kosunen TU. *Helicobacter pylori* and associated gastroduodenal disease. APMIS 1991;99:677-695.
16. Wyatt JJ. Histopathology of gastroduodenal inflammation: the impact of *Helicobacter pylori*. Histopathology 1995;26:1-15.
17. Eidt S, Stolte M. Prevalence of lymphoid follicles and aggregates in *Helicobacter pylori* gastritis in antral and body mucosa. J Clin Pathol 1993;46:832-835.

18. Genta RM, Hamner HW, Graham DY. Gastric lymphoid follicles in *Helicobacter pylori* infection: frequency, distribution, and response to triple therapy. *Hum Pathol* 1993;24:577-583.
19. Calvert R, Randerson J, Evans P, Cawkwell L, Lewis F, Dixon MF, Jack A, Owen R, Shiach C, Morgan GJ. Genetic abnormalities during transition from *Helicobacter-pylori*-associated gastritis to low-grade MALToma. *Lancet* 1995;345:26-27.
20. Karat D, O'Hanlon DM, Hayes N, Scott D, Rames SA, Griffin SM. Prospective study of *Helicobacter pylori* infection in primary gastric lymphoma. *B J Surg* 1995;82:1369-1370.
21. Miettinen A, Karttunen TJ, Alavaikko M. Lymphocytic gastritis and *Helicobacter pylori* infection in gastric lymphoma. *Gut* 1995;37:471-476.
22. Muller AF, Maloney A, Jenkins D, Dowling F, Smith P, Bessel EM, Toghil PF. Primary gastric lymphoma in clinical practise 1973-1992. *Gut* 1995;36:679-683.
23. Wotherspoon AC, Ortiz-Hidalgo C, Falzon MR, Isaacson PG. *Helicobacter pylori*-associated gastritis and primary B-cell gastric lymphoma. *Lancet* 1991;338:1175-1176.
24. Karnes WEJ, Samloff IM, Siurala M, Kekki M, Sipponen P, Kim SWR, Walsh JH. Positive serum antibody and negative tissue staining for *Helicobacter pylori* in subjects with atrophic body gastritis. *Gastroenterology* 1991;101:167-174.
25. Griffiths AP, Wyatt J, Jack AS, Dixon MF. Lymphocytic gastritis, gastric adenocarcinoma, and primary gastric lymphoma. *J Clin Pathol* 1994;47:1123-1124.
26. Shibata T, Imoto I, Ohuchi Y, Taguchi Y, Takaji S, Ikemura N, Nakao K, Shima T. *Helicobacter pylori* infection in patients with gastric carcinoma in biopsy and surgical resection specimens. *Cancer* 1996;77:1044-1049.
27. Osawa H, Inoue G, Yoshida Y. Inverse relation of serum *Helicobacter pylori* antibody titres and extent of intestinal metaplasia. *J Clin Pathol* 1996;49:112-115.
28. The EUROGAST Study Group. Epidemiology of, and risk factors for, *Helicobacter pylori* infection among 3194 asymptomatic subjects in 17 populations. *Gut* 1993;34:1672-1676.
29. Loffeld RJLF, Potters HVPJ, Arends JW, Stobberingh E, Flendrig JA, Van Spreeuwel JP. *Campylobacter* associated gastritis in patients with non-ulcer dyspepsia. *J Clin Pathol* 1988;41:85-88.
30. Wright DH. Lymphomas of mucosa-associated lymphoid tissue and antigen drive. *J Pathol* 1996;178:111-112.
31. Hussel T, Isaacson PG, Crabtree JE, Spencer J. The response of cells from low-grade B-cell gastric lymphomas of mucosa associated lymphoid tissue to *Helicobacter pylori*. *Lancet* 1993;342:571-574.

Chapter 5

***Helicobacter pylori* and non-*Helicobacter pylori* bacterial flora in gastric mucosal and tumour specimens of patients with primary gastric lymphoma**

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Summary

There is an association of *Helicobacter pylori* (*H.pylori*) with gastric mucosa-associated lymphoid tissue (MALT) and MALT-lymphoma. Histologically, mainly non-specific stains are used to detect *H.pylori*, such as Haematoxylin-Eosin (HE) or Modified Giemsa (MG).

In this study both a MG and a specific immunohistochemical staining (IMM) for *H.pylori* (DAKO B471) were performed on sequential slides of resection material containing tumour and non-tumorous gastric mucosa from patients with primary gastric lymphoma (n=52). Special attention was paid to the presence of non-*H.pylori* bacterial flora diagnosed by a positive MG (according to form and localisation) and a subsequently negative IMM. On all slides bacterial density was scored semi-quantitatively (grades 0,1,2,3).

In total 32 (61.5%) patients were *H.pylori*-positive by IMM and 34 (65.4%) were non-*H.pylori* positive by MG. In 24 of the 34 patients the non-*H.pylori* flora consisted mainly of cocci, in combination with rods in 15 patients, mostly in minor quantities; in another ten patients high numbers of both cocci and different types of rods were present. Most non-*H.pylori* bacteria were localized superficially, although in 22 patients minor quantities of non-*H.pylori* were seen in the glandular lumina also. Analysing all patients, no differences in density of *H.pylori* and of non-*H.pylori* flora were found. Only when comparing patients with a small to those with a large cell lymphoma, a significantly higher density of *H.pylori* was found in the corpus mucosa of large cell lymphomas and a higher prevalence of non-*H.pylori* was found in tumour, in antrum or corpus, of patients with large cell lymphomas.

In conclusion, using joint evaluation by Modified Giemsa and a *H.pylori*-specific immunohistochemical stain, the proportion of *H.pylori* positive gastric lymphoma patients was lower than in most previous publications and other bacteria were found in a relatively high proportion. The role of the non-*H.pylori* intragastric bacterial flora identified in this study has to be further elucidated with respect to the aetio-pathogenesis of primary gastric lymphoma.

Introduction

Helicobacter pylori (*H.pylori*) has been found in the gastric non-tumorous mucosa of 50% to more than 90% of patients with primary gastric lymphoma¹⁻⁴. A serological study of Parsonnet⁵ has shown that patients with gastric lymphoma have a significantly increased prevalence of previous *H.pylori* infection compared to matched controls. Furthermore, first results on eradication of *H.pylori* have demonstrated regression or disappearance of primary low grade gastric MALT lymphoma⁶⁻⁸.

In patients with gastric cancer an increased intragastric pH with an increased risk of bacterial overgrowth has been observed⁹⁻¹¹. Metabolic products of bacteria such as N-nitroso compounds, may contribute to the pathogenesis of the malignant process. It might be possible that a similar situation of reduced acidity and bacterial overgrowth occurs in patients with primary gastric lymphoma.

In histological studies of patients with primary gastric (MALT) lymphoma generally non-specific staining methods were used for the detection of *H.pylori* such as the Haematoxylin-Eosin or Modified Giemsa stains^{1,3,4}. In a previous study, we found a high specificity and sensitivity of an immunohistochemical stain for *H.pylori*¹², which has been confirmed by others¹³.

In this study we wanted to assess the presence and distribution of the bacterial flora in gastric tissue specimens of patients with primary gastric lymphoma by microscopy using a Modified Giemsa stain for the total bacterial flora (i.e. *H.pylori* and non-*H.pylori* bacterial flora), combined with a specific immunohistochemical stain for the detection of *H.pylori*.

Patients and Methods

Patients and specimens

Fifty-two patients (mean age 62.4 years; 31 male and 21 female) from ten hospitals in the provinces of Limburg and Brabant in the Netherlands with a documented primary gastric B-cell lymphoma were included in the study. For inclusion in this study, both tumour tissue and non-tumorous gastric mucosa had to be present in the surgically resected specimens (n=50) or endoscopic biopsies (n=2).

Histology

Serial sections of 4 μ m from formalin fixed and paraffin embedded specimens were cut for Haematoxylin-Eosin stain (HE), Modified Giemsa stain (MG) and an immunohistochemical stain (IMM), respectively. HE stained sections were revised for the classification of the primary gastric lymphoma and to assess the anatomical

tissue type (i.e. antral or corpus type) of the mucosa present in the specimens, which were used for the assessment of *H.pylori* and other bacteria. MG was performed as described by Gray¹⁴ to discover intragastric bacteria. For the IMM, a purified polyclonal antibody against *H.pylori* (DAKO B471, ITK Diagnostics BV, Uithoorn, The Netherlands) was applied. The antiserum was used in a 1:500 dilution. Further staining was performed with biotin-conjugated swine-anti-rabbit-antibodies (1:1000) (DAKO E431) as a second layer, followed by streptABC-HRP (1:1000) (DAKO K377). Sections were then stained with diaminobenzidine with 10% imidazol to intensify the reaction and were counterstained with haematoxylin. In the MG stain, the presence of *H.pylori* was scored according to the S-shaped form and the localisation on or within the mucus layer as well as within the crypts. The presence of *H.pylori* was only accepted if IMM on consecutive slides was positive. The presence of non-*H.pylori* flora was suspected in the MG stain by form and localisation of the bacteria and confirmed by negativity in the IMM. All slides were examined completely at 400x magnification. If necessary a 1000x magnification was used. Both *H.pylori* and non-*H.pylori* were scored semi-quantitatively and graded as follows: no bacteria found, grade 0; occasionally bacteria found, grade 1; scattered bacteria found in many high power fields, grade 2; and numerous bacteria found in most high power fields, grade 3¹⁵.

Analysis

Prevalence and density of *H.pylori* and non-*H.pylori* were compared in total and according to their presence in tumour tissue or non-tumorous antral or corpus type mucosa. Differences in prevalence were analyzed using a Chi-Square test with Fisher's Exact test when necessary. Differences in density of *H.pylori* or non-*H.pylori* positive specimens were analyzed with a Mann-Whitney U test. The prevalence and density of *H.pylori* and non-*H.pylori* in antrum compared to corpus and in tumour tissue compared to mucosa within the patients, were analyzed by paired non-parametrical tests as well. A p-value of less than 0.05 was considered to be significant. Differences in the sex distribution and lymphoma type (small cell versus large cell gastric lymphoma) between different patient subgroups were analyzed with the Chi-Square test. Differences in age between the different groups were compared using the Mann-Whitney U test or Kruskal-Wallis one-way Anova, depending on the number of groups to be compared.

Results

Of 52 patients included, 17 had a small cell and 35 a large cell primary gastric lymphoma according to the criteria given by Isaacson^{16,17}. The diagnosis of MALT

could be confirmed in 43 of 52 patients. The remaining nine large cell lymphomas could not further be classified as being from MALT origin because of the absence of a small cell component. Besides tumour tissue, normal appearing antral or corpus mucosa, or both types of mucosa were present for 9, 8, and 35 patients, respectively.

Prevalence of *H.pylori* and non-*H.pylori* per patient

H.pylori could be detected by IMM in 32 of the 52 (61.5%) patients. Using MG 36 of 52 (69.2%) patients were scored *H.pylori* positive. Non-*H.pylori* flora was detected in 34 of 52 (65.4%) patients by MG. *H.pylori* and non-*H.pylori* were present simultaneously in 19 of 52 (36.5%) patients, *H.pylori* only in 13 (25.0%) and non-*H.pylori* flora only in 15 patients (28.8%). In five (9.6%) patients no bacteria were found in any of the tissue specimens.

H.pylori was only detected in the non-tumorous gastric mucosa present in the specimens, in contrast to non-*H.pylori* flora which was found on and within superficial tumour tissue as well as in the surrounding gastric mucosa.

For further analysis as to the prevalence and density of *H.pylori*, IMM results were considered as the standard for the detection of *H.pylori* because of the proven high specificity and sensitivity of IMM^{12,13}.

Description of non-*H.pylori* flora

In 24 of 34 patients the non-*H.pylori* flora consisted mainly of cocci, 15 times in combination with rods. The density of cocci was higher than that of rods in nine of them. In the other ten patients, massive numbers of both cocci (grade 3) and of different types of rods (grade 3) were found. When several resection specimens were available from one patient, the bacterial forms observed were similar for each patient.

In tumour tissue the non-*H.pylori* flora was localized on the surface of and within the superficial layer of the tumour. When non-*H.pylori* flora was observed in the gastric mucosa, it was mainly localized superficially (in strains of mucus overlying the mucosa), although in 22 patients minor numbers were also seen on or within the mucus layer of the glands. In four patients coccoid bacteria were also present between *H.pylori* bacteria in the mucus layer of the glands. This will be coccoid forms of *H.pylori*, as they were found to be IMM positive.

In addition to the bacteria observed, in 16 and 9 patients, respectively, yeast cells (median density grade 2) and mycelium and/or pseudo-mycelium threads (median density grade 3) (confirmed by Grocott stain) were seen, predominantly in the tumour area.

Prevalence and density of *H.pylori* and non-*H.pylori* in antrum and corpus.

In table 5.1 the prevalence of *H.pylori* (by IMM) and non-*H.pylori* (by MG) in antrum and corpus specimens is given for all patients. Figure 5.1 shows the frequency of grade 1, 2, and 3 as percentage of *H.pylori* or non-*H.pylori* positive specimens per anatomical site. Per patient the median gradation per anatomical site was used for the analyses of the density, if more than one specimen was available. When the median value was lower than the 75th percentile of the gradation, we have chosen a "worst case" approach and used the 75th percentile.

Table 5.1 Prevalence of *H.pylori* and non-*H.pylori* in antral and corpus specimens.

Anatomical site		n	<i>H.pylori</i> positive	non- <i>H.pylori</i> positive
Antrum	mucosa	44	20 (45.5%)	24 (54.5%)
	tumour	36	-	19 (52.8%)
Corpus	mucosa	43	27 (62.8%)	21 (48.8%)
	tumour	35	-	18 (51.4%)

A tendency ($p=0.06$) was observed concerning a higher prevalence of *H.pylori* in corpus (62.8%) compared to antral type mucosa (43.2%). No significant differences were found in the density of *H.pylori* positive antrum or corpus specimens.

Non-*H.pylori* flora was found in antral mucosa of 24 of 44 patients (54.5%) and in corpus type mucosa of 21 of 43 patients (48.8%). No significant differences were found in either the prevalence or the density of this non-*H.pylori* flora in antral or corpus type mucosa. Moreover, both the prevalence and density of non-*H.pylori* flora were similar on or within tumour tissue in antrum (50.0%) or corpus (51.4%) compared to the normal appearing antral (54.5%) or corpus (48.8%) mucosa, respectively. Comparing the presence of *H.pylori* with that of non-*H.pylori* in antrum or corpus, both types of flora were found with a similar frequency and density.

Among the patients with primary gastric lymphoma, four groups can be distinguished according to the bacterial floras detected: those with *H.pylori* only (group 1, $n=13$), with non-*H.pylori* only (group 2, $n=15$), with both *H.pylori* and non-*H.pylori* (group 3, $n=19$) and those where no bacteria could be detected in any of the specimens (group 4, $n=5$).

Figure 5.1 Density of *H.pylori* and non-*H.pylori* flora in antrum and corpus mucosa or tumour tissue (Percentage of grade 1,2,3 of total number of positives).

Table 5.2 Sex distribution, age and lymphoma type (small vs large cell lymphoma) of patients according to the presence of *H.pylori* (Hp) and/or non-*H.pylori* (nHp) bacterial flora.

	Hp only gr 1, n=13	nHp only gr 2, n=15	both Hp/nHp gr 3, n=19	no flora gr 4, n=5
Age (median, range)	56 (26-79) ¹	60 (50-92)	64 (35-83)	72 (61-87) ¹
Sex (male/female)	10/3 ²	6/9 ²	12/7	3/2
Lymphoma type (small/large cell)	7/6	3/12	6/13	1/4

¹Significant higher age in group 4 versus group 1 (Mann-Whitney, $p < 0.05$)

²Significant more females in group 2 versus group 1 (Chi-square, $p < 0.05$)

No statistical differences were observed in the distribution of small cell versus large cell gastric lymphoma between the four groups, although a trend was found for a higher number of large cell lymphomas to be present in group 2 ($p=0.07$) and group 3 ($p=0.07$) compared with group 1. The age of patients in group 4 was significantly higher than of group 2 ($p=0.04$) and in group 2 significantly more females were present compared with group 1 ($p=0.048$). No further differences concerning gender and age between the four groups were found (Table 5.2). Comparing the presence of *H.pylori* in group 1 with group 3, or the presence of non-*H.pylori* in group 2 with group 3, no significant differences were found in the prevalence or density of *H.pylori* or non-*H.pylori* in antrum or corpus. Also within each group no differences were observed comparing antrum with corpus and comparing *H.pylori* with non-*H.pylori* (Table 5.3, figure 5.2).

Table 5.3 Prevalence of *H.pylori* and non-*H.pylori* in antrum and corpus of patients with *H.pylori* only, non-*H.pylori* only or both types of flora.

		Hp only positive/n (%)	nHp only positive/n(%)	both Hp/nHp positive/n(%)
<i>H.pylori</i>				
antrum	mucosa	9/10 (90.0)	--	11/15 (73.3)
corpus	mucosa	11/12 (91.7)	--	16/17 (94.1)
<i>non-H.pylori</i>				
antrum	mucosa	--	11/14 (78.6)	13/15 (86.7)
	tumour	--	10/12 (83.3)	9/13 (69.2)
corpus	mucosa	--	6/11 (54.4)	15/17 (88.2)
	tumour	--	7/8 (87.5)	11/16 (68.8)

***H.pylori* and non-*H.pylori* in small versus large cell gastric lymphoma**

In total 17 patients had a small (10 male/7 female, median age 55 (26-83) years), and 35 patients had a large cell gastric lymphoma (21 male/14 female, median age 67 (35-92) years).

No significant differences were found in the distribution of males and females in both groups. However, patients with a large cell lymphoma were significantly older (Mann-Whitney, $p=0.03$) than those with a small cell lymphoma.

The prevalence of *H.pylori* was similar in antrum and corpus mucosa of patients with small cell versus patients with large cell lymphomas (Table 5.4). The density of *H.pylori* was significantly higher ($p=0.009$) in corpus mucosa of patients with a large cell compared with those with a small cell lymphoma (Figure 5.3).

Regarding the non-*H.pylori* flora, the prevalence was higher in tumour of antrum or corpus in patients with a large compared to those with a small cell lymphoma (Table 5.4) (antrum, $p=0.02$; corpus, $p=0.03$). Further differences in the prevalence or density of non-*H.pylori* were not found.

Figure 5.2 Density of *H. pylori* (A) or non-*H. pylori* in gastric mucosa (B) and tumour tissue (C) of patients with only *H. pylori* (group 1) or only non-*H. pylori* (group 2) compared with patients with both *H. pylori* and non-*H. pylori* (group 3).

Figure 5.3 Density of *H.pylori* and non-*H.pylori* flora in antrum en corpus of patients with a small cell (A) or large cell primary gastric lymphoma (B) (Percentage of grade 1,2,3 of total number of positives).

Table 5.4 Prevalence (%) of *H.pylori* (by IMM) and non-*H.pylori* (by MG) in patients with small or large cell gastric lymphoma.

	Small cell (n=17) pos/n (%)	Large cell (n=35) pos/n (%)
Hp antrum muc	7/14 (50.0%)	13/30 (43.3%)
Hp corpus muc	12/16 (75.0%)	15/27 (55.0%)
nHp antrum muc	6/14 (42.9%)	18/30 (60.0%)
nHp antrum tum ¹	3/12 (25.0%)	16/24 (66.7%)
nHp corpus muc	7/16 (43.8%)	14/27 (51.9%)
nHp corpus tum ²	4/14 (28.6%)	14/21 (66.7%)

muc: mucosa present in antrum or corpus; tum: tumour tissue present in antrum or corpus

1) Chi-Square, $p=0.02$; 2) Chi-Square, $p=0.03$

Discussion

Using a joint evaluation with the non-specific MG stain for the total intragastric bacterial flora and a specific immunohistochemical stain for the detection of *H.pylori*, non-*H.pylori* flora was detected in 67.3% and *H.pylori* in 61.5% of patients with primary gastric lymphoma. Both types of bacterial flora were simultaneously present in 36.5% of patients. *H.pylori* was only found in non-tumorous gastric mucosa, whereas non-*H.pylori* flora was present in antrum and corpus mucosa as well as on and within the surface of the lymphomas.

In this study using a specific immunohistochemical stain, fewer patients with primary gastric lymphoma were found to be *H.pylori*-positive compared to other studies^{1,3,18}. An explanation for this discrepancy could be that the use of MG and HE has overestimated the *H.pylori* frequency in comparison with the *H.pylori* specific IMM stain as used in this study. Secondly, it cannot be excluded that real differences in the prevalence of *H.pylori* exist between different geographical regions as described by Doglioni¹⁹. Furthermore, it is possible that *H.pylori*-negative patients at present have previously been positive, as it has been described in patients with lymphocytic gastritis^{20,21}. Earlier *H.pylori*-positivity can theoretically be controlled for by serology. Unfortunately, it was not possible to assess sero-positivity for *H.pylori* in this study as we performed a retrospective study on histological material only derived from ten different hospitals.

As the archival material of this study was obtained before 1992. *H.pylori* eradication therapy was unlikely to be generally applied. Eradication is therefore not the cause of the low *H.pylori* prevalence found in this study. Relatively low *H.pylori* prevalence in patients with primary gastric lymphoma have recently also

been described by others^{2,4}.

The prevalence of intragastric *H.pylori*-positivity in this study is not much different from that found in patients with non-ulcer dyspepsia or in healthy blood donors of the same geographical region of a similar age, where *H.pylori* was present in 49.6% and 58.3%, respectively, using serology²². This has to be taken into account for the discussion of the etiological role of *H.pylori* in gastric lymphoma.

To our knowledge, this is the first study to focus on the presence of non-*H.pylori* flora besides *H.pylori* in primary gastric lymphoma. Non-*H.pylori* flora occurred in a surprisingly high proportion of patients with a primary gastric lymphoma. Several explanations for this phenomenon can be put forward:

- Colonization with non-*H.pylori* flora can be a consequence of a decreased gastric acid secretion due to atrophic gastritis. Atrophy of the gastric body has been described in lymphocytic gastritis which is common in patients with primary gastric lymphoma^{21,23}.
- The tumour itself can cause decreased acid secretion by involvement of acid secreting gastric mucosa, and altered gastric motility as a consequence of tumour infiltration can lead to a stagnant loop syndrome with bacterial overgrowth. Theoretically, the non-*H.pylori* flora found in non-tumorous gastric mucosa could be spreading out from the tumour area.
- Finally, patients with primary gastric lymphoma might have gastrointestinal complaints and may have been treated with gastric acid inhibitors, resulting in an increased intragastric pH and bacterial overgrowth^{24,25}.

Hitherto, little attention has been paid to the distribution of *H.pylori* and other bacteria in the stomach of lymphoma patients. In this patient group the prevalence of *H.pylori* was somewhat higher in corpus than in antral type mucosa (although not significant). A similar situation is found during acid inhibitory treatment for *H.pylori* associated peptic disorders, where a displacement of *H.pylori* from antrum towards corpus can occur^{26,27}. The finding of a trend of a greater number of *H.pylori* positive corpus- than antrum-specimens in patients with primary gastric lymphomas can support the presence of a reduced intragastric acidity in the present study population. The *H.pylori* density in corpus mucosa of patients with large cell lymphomas is significantly higher than in patients with small cell lymphomas. This may indicate that there can be a more prolonged or stronger reduction in gastric acidity in the former group of patients. In addition, this patient group is significantly older than those with a small cell lymphoma and therefore a longer duration of the *H.pylori* infection with subsequent atrophy may exist.

For non-*H.pylori* bacteria no difference in distribution was found between antrum and corpus or between tumour tissue and gastric mucosa analysing all patients. This homogeneous distribution of non-*H.pylori* flora in the stomach supports the presence of hypochlorhydria or achlorhydria in the stomach as an explanation for the presence of these microorganisms. However, a significantly higher prevalence

of non-*H.pylori* was found in tumour tissue of patients with a large cell compared with small cell lymphoma.

H.pylori and non-*H.pylori* bacterial flora were both found with a similar frequency, density and distribution. No differences were found in the subgroups of patients with *H.pylori* only, non-*H.pylori* only, and the joint occurrence of *H.pylori* and non-*H.pylori*. This suggests that there is no direct relation between the two types of flora and that they may be independent from each other.

The role of the non-*H.pylori* bacteria in relation to primary gastric lymphoma is not clear yet. To our knowledge only the study of Blackburn²⁸ has some relation to this subject; in that study an increased mortality from both gastric cancer and leukaemia was found in patients with pernicious anaemia, a condition known to be associated with intragastric bacterial overgrowth^{29,30}.

On the one hand, the non-*H.pylori* flora could be a consequence of the functional situation in gastric lymphoma patients, resulting from the stagnant loop situation and/or reduced gastric acidity. On the other hand, this flora may also play a role in the development of the lymphomas by being an antigenic stimulus for an inflammatory response. In parallel to gastric cancer, this bacteria could also be responsible for the production of toxic or carcinogenic metabolites, such as N-nitroso-compounds¹¹. The higher prevalence of non-*H.pylori* flora in tumour tissue of large cell compared with small cell lymphomas may be a consequence of differences in tumour mass and/or gastric acidity between both groups as well as the higher age of the former group of patients. However, it cannot be excluded that the non-*H.pylori* flora contributes to the development of a large cell lymphoma. Further studies on the role of the non-*H.pylori* flora in the development and progression of small and large cell primary gastric lymphoma are therefore required.

Independent of the possible pathogenetic role of the non-*H.pylori* flora found in gastric lymphoma, the high frequency and concentrations of this flora in patients with primary gastric lymphoma may interfere with the detection of *H.pylori* and possibly lead to overestimation of *H.pylori*¹². We therefore recommend the use of a *H.pylori*-specific immunohistochemical staining for *H.pylori* for further studies on the role of this bacterium in the pathogenesis and therapy of gastric MALT-lymphomas.

References

1. Wotherspoon AC, Ortiz-Hidalgo C, Falzon MR, Isaacson PG. *Helicobacter pylori*-associated gastritis and primary B-cell gastric lymphoma. *Lancet* 1991;338:1175-1176.
2. Fischbach W, Kolve ME, Dragosics B and the German-Austrian Gastrointestinal Study Group. Prevalence of *Helicobacter pylori* infection in primary gastric lymphoma of the MALT: There is a difference between low grade and high grade lymphoma. *Gut* 1995;37(suppl 2):A75
3. Muller AF, Maloney A, Jenkins D, Dowling F, Smith P, Bessell EM, Toghill PJ. Primary gastric lymphoma in clinical practice 1973-1992. *Gut* 1995;36:679-683.
4. Karat D, O'Hanlon DM, Hayes N, Scott D, Raimes SA, Griffin SM. Prospective study of *Helicobacter pylori* infection in primary gastric lymphoma. *Br J Surg* 1995;82:1369-1370.
5. Parsonnet J, Hansen S, Rodriguez L, Gelb AB, Warnke RA, Jellum E, Orentreich N, Vogelstein JN, Friedman O. *Helicobacter pylori* infection and gastric lymphoma. *N Engl J Med* 1994;330:1267-1271.
6. Wotherspoon AC, Doglioni C, Diss TC, Pan L, Moschini A, de Boni M. Regression of primary low-grade B-cell gastric lymphoma of mucosa-associated lymphoid tissue type after eradication of *Helicobacter pylori*. *Lancet* 1993;342:575-577.
7. Weber DM, Dimopoulos MA, Anandu DP, Pugh WC, Steinbach G. Regression of gastric lymphoma of mucosa-associated lymphoid tissue with antibiotic therapy for *Helicobacter pylori*. *Gastroenterol* 1994;107:1835-1838.
8. Roggero E, Zucca E, Pinotti G, Pascarella A, Capella C, Savio A, Pedrinis E, Paterlini A, Venco A, Cavalli F. Eradication of *Helicobacter pylori* infection in primary low-grade gastric lymphoma of mucosa-associated lymphoid tissue. *Ann Intern Med* 1995;122:767-769.
9. Kuster G, Remine W, Dockerty M. Gastric cancer in pernicious anemia and in patients with and without achlorhydria. *Ann Surg* 1972;175:783-789.
10. Muscroft TJ, Deane SP, Youngs D, Burdon DW, Keighley MRB. The microflora of the postoperative stomach. *Br J Surg* 1981;86:560-564.
11. Houben GMP and Stockbrügger RW. Bacteria in the aetio-pathogenesis of gastric cancer: A review. *Scand J Gastroenterol* 1995;30(suppl 212):13-18.
12. Jonkers D, Houben P, Stobberingh E, Stockbrügger R. Ongoing gastric acid inhibition is a confounding factor in *Helicobacter pylori* diagnosis. *Eur J Gastroenterol Hepatol* 1997;9:49-53.
13. Few MJ, Codd J, Marker AJ. Grading *Helicobacter pylori* infection in gastric biopsies. *J Pathol* 1996;178(suppl):16A.
14. Gray SF, Wyatt JL, Rathbone BJ. Simplified techniques for identifying *Campylobacter pyloridis*. *J Clin Pathol* 1986;39:1279.
15. Marshall BJ, Warren JR. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. *Lancet* 1984;i:1311-1314

16. Isaacson PG, Spencer J. Malignant lymphoma of mucosa-associated lymphoid tissue. *Histopathol* 1987;11:445-462.
17. Isaacson PG. Lymphomas of mucosa-associated lymphoid tissue (MALT). *Histopathol* 1990;16:617-619.
18. Eidt S, Stolte M. Evidence of a malignant cycle in early gastric lymphoma. *Endoscopy* 1994;26:299-302.
19. Doglioni C, Wotherspoon AC, Moschini A, de Boni M, Isaacson PG. High incidence of primary gastric lymphoma in northeastern Italy. *Lancet* 1992;339:834-835.
20. Karnes WE, Samloff IM, Siurala M, Kekki M, Sipponen P, Kim SW, Walsh JM. Positive serum antibody and negative tissue staining for *Helicobacter pylori* in subjects with atrophic body gastritis. *Gastroenterol* 1991;101:167-174.
21. Niemelä S, Karttunen T, Kerola T, Karttunen R. Ten year follow up study of lymphocytic gastritis: further evidence on *Helicobacter pylori* as a cause of lymphocytic gastritis and corpus gastritis. *J Clin Pathol* 1995;48:1111-1116.
22. Loffeld RJLF, Stobberingh E, Van Spreuwel JP, Flendrig JA, Arends JW. The prevalence of anti-*Helicobacter* (*Campylobacter*) antibodies in patients and healthy blood donors. *J Med Microbiol* 1990;32:105-109.
23. Miettinen, A, Karttunen TJ, Alavaikko M. Lymphocytic gastritis and *Helicobacter pylori* infection in gastric lymphoma. *Gut* 1995;37:471-474.
24. Stockbrügger RW, Cotton PB, Eugenides N, Bartholomew BA, Hill MJ, and Walters CL. Intra gastric nitrites, nitrosamines, and bacterial overgrowth during cimetidine treatment. *Gut* 1982;23:1048-1054.
25. Verdu E, Viani F, Armstrong D, Fraser R, Siegrist HH, Pignatelli B, Idstrom JP, Cerderberg C, Blum AL, Fried M. Effect of omeprazole on intra gastric bacterial counts, nitrates, nitrites, and N-nitroso compounds. *Gut* 1994;35:455-460.
26. Kuipers EJ, Uytterlinde AM, Pena AS et al. Increase of *Helicobacter pylori*-associated corpus gastritis during acid suppressive therapy: Implications for long-term safety. *Am J Gastroenterol* 1995;90:1401-1406.
27. Logan RPH, Walker MM, Misiewicz JJ, Gummatt PA, Karim QN, Baron JH. Changes in the intra gastric distribution of *Helicobacter pylori* during treatment with omeprazole. *Gut* 1995;36:12-16.
28. Blackburn EK, Callender SI, Dacie JV, Doll R, Giirdwood RH, Mollin DL, Saracci R, Stafford JL, Thompson RB, Varadi S, Wetherley-Mein G. Possible association between pernicious anaemia: a prospective study of 1625 patients with a note on the very high incidence of stomach cancer. *Int J Cancer* 1968;3:163-170.
29. Dolby JM, Webster ADB, Borriello SP, Barclay FE, Bartholomew BA, Hill MJ. Bacterial colonization and nitrite concentration in the achlorhydric stomachs of patients with primary hypogammaglobulinaemia or classical pernicious anaemia. *Scand J Gastroenterol* 1984;19:105-110.
30. Stockbrügger RW, Cotton PB, Menon GG, Beilby JOW, Bartholomew BA, Hill MJ, Walters CL. Pernicious anaemia, intra gastric bacterial overgrowth and possible consequences. *Scand J Gastroenterol* 1984;19:355-364.

Chapter 6

In situ hybridisation studies in small and large cell gastric MALT lymphoma demonstrate higher numbers of numerical chromosomal aberrations in large cell lymphomas

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Will be submitted in an adapted version together with a Leiden study Group on malignant lymphoma

Summary

Background

Several histological subtypes of non-Hodgkin's lymphomas bear specific chromosomal abnormalities. In low grade MALT lymphoma, a trisomy of chromosome three has recurrently been detected. In the current study, we analyzed both small and large cell gastric MALT lymphomas for numerical chromosomal aberrations.

Materials and methods

Seven small cell MALT lymphomas and thirteen large cell MALT lymphomas of the stomach were analyzed for numerical chromosomal aberrations using in situ hybridisation (ISH). Nuclei were isolated from formalin-fixed, paraffin-embedded tissues and cytospinned into slides, α -Satellite centromeric probes hybridising to the pericentromeric regions of chromosomes 1, 3, 7, 8, 9, 11, 12, 15, 17 and 18 were used. Biotin- and digoxigenin-labelled probes were detected and peroxidase activity was eventually visualized with diaminobenzidine. At least 300 nuclei per case were evaluated for the number of ISH signals.

Results

Seventeen ISH preparations expressed more than 10% of nuclei with more than two ISH signals, which was used as a cut-off level. ISH procedures for chromosomes 8, 9 and 11 were often unsuccessful. A trisomy for chromosome 12 was the most often observed abnormality (in six cases), followed by trisomy 18 (3 cases), trisomy 15 (2 cases) and trisomy 7 (2 cases). Large cell cases more frequently showed aberrations when compared to small cell cases (61.5% vs 42.8%). In both, trisomy 12 was the most frequent abnormality. Large cell cases more often showed numerical abnormalities for more than one chromosome. Trisomy 3 was detected in only one (small cell) case.

Conclusion

Numerical chromosomal abnormalities are not common in small and large cell gastric MALT lymphoma. Although small and large cell lymphomas in general showed abnormalities for the same chromosomes, large cell lymphomas showed more, and also abnormalities for more than one chromosome per case compared to small cell lymphomas. This may represent increasing genetic instability in large cell lymphomas. We could not confirm earlier findings of a recurrent trisomy 3 in MALT lymphoma.

Introduction

Non-Hodgkin's lymphomas are known to bear chromosomal abnormalities which, in part, can be related to their histological subtype as well as immunophenotype¹⁻⁷. The growing knowledge of genetic aberrations in lymphoma already had impact on classification⁸ so that data on chromosomal aberrations have now been included in the most recent REAL classification, which combines histopathological and clinical information with genetic aberrations to distinguish lymphoma subtypes^{9,10}.

The most traditional technique to study abnormal chromosomal constellation was karyotyping which required culture of cells to obtain metaphase spreads. The development of in situ hybridization on interphase nuclei in combination with the development of pretreatment protocols for paraffin-embedded tissues makes chromosomal information available in archival material which allows analysis of material of patients with a known clinical outcome¹¹⁻¹⁴.

In 1983 Isaacson introduced primary gastric lymphoma of mucosa-associated lymphoid tissue (MALT) as a distinct entity¹⁵. Since then, several studies have addressed the question whether these lymphomas are indeed a separate clinicopathological entity, using several approaches, like for example clinical differences between MALT lymphomas and non-MALT lymphomas. Cytogenetic studies also play an important role in analysing this question, like the finding of trisomy 3 in 60% of small cell MALT lymphomas by Wotherspoon and colleagues¹⁶.

To our knowledge, study of numerical chromosomal abnormalities in primary gastric lymphoma has mainly focused on low-grade MALT lymphoma and scarcely on high-grade primary gastric lymphoma¹⁷. Whether these more commonly occurring large cell neoplasms are derived from low-grade precursors or if they develop *de novo* in the stomach is still disputed. Therefore it would be interesting to collect information on the chromosomal constitution of gastric lymphoma in general and more specifically on large cell gastric lymphoma. In the present study we investigated numerical chromosomal abnormalities using α -satellite-centromeric DNA probes for chromosomes 1,3,7,8,9,11,12,15,17 and 18.

Materials and methods

Materials

Formalin-fixed and paraffin-embedded archival tissues of primary gastric B-cell lymphoma were analyzed by single target ISH. Cases were classified as small cell primary gastric lymphoma of MALT (n=7) and large cell primary gastric lymphoma of MALT (n=13), using the criteria described by Isaacson^{15,18}. Large

cell lymphomas were defined by the presence of sheets of large blastic cells. A MALT origin was defined by the presence of a small cell component showing the typical features of MALT: reactive follicles, surrounded by centrocyte like cells, plasma cell differentiation and lymphoepithelial lesions. Four cases of chronic active gastritis associated with *H. pylori* were used as biological control tissues. Paraffin-embedded formalin-fixed reactive lymph node specimens were also used as controls.

Isolation of nuclei

From these tissues nuclei were isolated by a method modified from Schutte et al¹⁹. Shortly, 2 sections of 30µm were made, and using a serial haematoxylin-eosin stained thin section, tumour areas were selected. The sections were deparaffinized in xylol and rehydrated in graded ethanols. Subsequently, they were pretreated by incubation for 20 minutes in 85% formic acid/0.3% H₂O₂, and rinsed in 0.01N HCl. Enzymatical digestion was performed with 4 mg/ml pepsin in 0.2N HCl for 30 minutes at 37°C. The digest was cytopinned for 5 minutes at 700 rpm onto Superfrost slides and air-dried. The slides were dehydrated in 70% ethanol/0.01N HCl to preserve nuclear morphology, and subsequently further dehydrated and air-dried.

In situ hybridization

DNA probes which hybridize to the (peri)centromeric regions of the following human chromosomes were used: 1 (pUC1.77, 1.77kb)²⁰, 3 (D3Z1²¹ and pAE0.68, 0.68kb, Boehringer Mannheim), 7 (p7t1, 0.68kb)²², 8 (D8Z2, 2.55kb)²³, 9 (pHUR98, 0.16kb)²⁴, 11 (pLC11A, 0.85kb)²⁵, 12 (p12H8, 1.35kb)²⁶, 15 (D15Z1, 1.80kb)²⁷, 17 (p17H8, 2.7kb)²⁸, and 18 (L1.84, 0.68kb)²⁹. All probes except probe pAE0.68 for chromosome 3, which was digoxigenin-labelled, were labelled with biotin-11-dUTP (Sigma).

These probes were diluted in hybridization buffer (60% formamide, 2xSSC pH 7.0, 10% dextranulphate, 0.2 µg/µl herring sperm DNA and 0.2 µg/µl yeast tRNA) to a final concentration of 0.2 ng/µl for pUC1.77 and 1 ng/µl for the other DNA probes.

Probe and target DNA were denaturated at 80°C for 5 minutes (10 µl of hybridization mixture added to the slides under a coverslip), followed by overnight hybridization at 37°C.

Subsequently, samples were washed 2x5 minutes in 2xSSC/0.05% Tween-20 at 42°C, 2x5 minutes in 0.1xSSC at 60°C and 2x5 minutes in 4xSSC-0.05% Tween-20 at room temperature. Detection of the biotinylated probes was performed with

mouse anti-biotin (1:100), detection of the digoxigenin-labelled probe for chromosome 3 was performed with mouse anti-digoxigenin (1:2000). This step was followed by the application of biotinylated horse-anti-mouse and avidin-biotin-peroxidase for all slides. All incubations were performed for 30 minutes in a humidified chamber at 37°C. When necessary, signals were amplified using CARD (Catalysed Reporter Deposition)^{30,31}: following washing of the last step (avidin-biotin-peroxidase), biotinylated tyramide was applied to the slide and was afterwards detected by Avidin-Peroxidase. The peroxidase activity was visualized with diaminobenzidine (DAB) and nuclei were counterstained with haematoxylin.

Evaluation

Evaluation of the in situ hybridisation signals was performed using the criteria described by Hopman et al.³² which include the following criteria: nuclei should not overlap, ISH signals should have more or less the same homogeneous intensity, minor hybridisation spots (recognisable by lower intensity) were not counted and paired (split) spots were counted as one chromosome complement. Three to four hundred nuclei per case were examined for the number of signals at a magnification of 400X or when necessary at 1000X under oil immersion. The relative numbers of cells showing zero, one, two, three, or more spots were expressed as percentages.

Results

Efficiency of the hybridisation procedures showed differences for the tested chromosomes. The ISH procedure was relatively inefficient using probes for chromosome 8,9,11. As described in the materials and methods, two probes were used for the detection of chromosome 3. Only in situ hybridisation using the dig-labelled pAE0.68 probe lead to evaluable signals, whereas the other probe gave very weak signals. Both easily showed minor hybridisation spots. Using a cut-off level of more than 10% nuclei with more than two signals per nucleus, 17 ISH preparations showed numerical aberrations. Figure 6.1 shows some examples of this technique. Figure 6.2 shows the aberrations per chromosome and table 6.1 shows the aberrations in the individual lymphoma cases. Using this cut-off level, the most frequently found aberration was a trisomy for chromosome 12 (6 cases), followed by trisomy 18 (3 cases), trisomy 15 (2 cases) and trisomy 7 (2 cases). Trisomy 1, 3, 11 and 17 were all seen in one case.

In general, large cell lymphomas showed more aberrations than the small cell variants. Three out of seven small cell cases (42.8%) showed chromosomal aberrations. Two of these cases showed trisomy for only one chromosome, one

case showed a trisomy for both chromosomes 11 and 12. The most frequent abnormality was trisomy of chromosome 12, in 2 cases. Trisomy 3 was seen in one case.

Table 6.1 Results per case in small and large cell primary gastric lymphoma (D=disomy, X=trisomy, -=not evaluable).

Case	SC/LC	Chromosome number									
		1	3	7	8	9	11	12	15	17	18
8610044	SC	D	X	D	-	-	-	D	D	D	D
863415	SC	D	D	D	-	-	-	D	D	D	-
871277	SC	D	D	D	D	-	-	D	D	D	D
915995	SC	D	D	D	D	D	D	D	D	D	D
89199	SC	D	D	D	D	D	X	X	D	D	D
8922470	SC	D	D	D	D	-	-	X	D	D	D
9011070	SC	D	-	D	D	-	-	D	D	D	D
Total	SC	0	1	0	0	0	1	2	0	0	0
862829	LC	D	-	D	D	-	-	D	D	D	X
872133	LC	D	D	D	-	-	-	D	D	D	D
913198	LC	D	D	D	D	-	-	D	D	D	D
878591	LC	D	D	D	D	D	-	D	X	D	D
90172	LC	D	D	D	D	-	-	X	D	D	X
90732	LC	D	-	D	D	-	-	D	D	D	-
912958	LC	D	D	X	D	-	-	D	D	D	X
922181	LC	X	D	D	-	-	D	-	X	D	D
926396	LC	D	D	X	-	-	-	X	D	X	D
8155010	LC	D	-	D	D	-	D	D	D	D	D
8211842	LC	D	D	-	D	D	-	D	D	D	D
8316589	LC	D	D	D	D	-	-	X	D	D	D
8322923	LC	D	D	D	D	D	D	X	D	D	D
Total	LC	1	0	2	0	0	0	4	2	1	3

Eight out of 13 large cell cases (61.5%) showed numerical aberrations. Four cases showed aberrations for more than one chromosome, in four cases only one chromosome showed a numerical gain. In the large cell lymphomas, like in the small cell lymphomas, trisomy 12 was the most often detected abnormality, in four cases. Trisomy 18 was detected in three cases and trisomy 7 and 15 were seen in two cases.

Figure 6.2 Percentage of nuclei showing three ISH signals for all the chromosomes tested. (Line represents cut-off level of more than 10% aberrant nuclei).

Discussion

Specific chromosomal changes have been found in various subtypes of non-Hodgkin's lymphoma, like rearrangements, gains, or losses of chromosomal segments. These changes often point to loci in the genome with specific genes *important in tumorigenesis*. The present study was conducted to analyze chromosomal aberrations in primary small and large cell gastric lymphoma. The relationship between these entities is not yet elucidated, however, there are indications that large cell lymphomas may evolve from their small cell precursors. Among these, clonal relationships have been shown^{33,34}.

If large cell lymphomas progress from small cell MALT lymphomas it would be expected that large cell lymphomas bear at least the same chromosomal constitution (i.e. aberrations), apart from additionally acquired abnormalities. Indeed, part of our data may point to such a progression, i.e. the presence of trisomy 12 in both small and large cell lymphomas, however, only present in a low frequency of cases.

Furthermore, the finding of relatively few, and in general only aberrations for one chromosome in small cell lymphomas contrasts with data in large cell lymphomas. Large cell lymphomas showed more and also more different chromosomal aberrations per case. These findings support the idea of genetic instability in

tumours, with the accumulation of genetic aberrations in transformation from low to high-grade malignancy.

In the past, cytogenetic analysis in primary lymphomas of MALT has been hampered by their poor *in vivo* growth, and moreover very few fresh tumour tissue is generally available. The first studies investigating cytogenetics in MALT lymphoma date from 1992^{35,37} and found structural aberrations like translocations t(1;14) and t(11;18) and numerical aberrations being a recurrently noticed gain of chromosome 3. Whang-Peng and colleagues studied two cases with MALT lymphoma and also found that aberrations of chromosomes 3, 7 and 12 were the most common findings³⁸. The finding of the same translocation t(1;14) in a low-grade primary MALT lymphoma of the lung 2 years earlier supported the idea of MALT lymphoma as a separate entity³⁹. Only few high-grade cases have been studied¹⁷, but the presence of more abnormal karyotypes in these lymphomas compared to their low-grade counterparts supported the role of chromosomal aberrations in tumour progression.

The development of *in situ* hybridisation in interphase cells overcomes the earlier mentioned problems. This enables investigation of larger series of these lymphomas in retrospect. Wotherspoon et al. used this technique to analyze 70 cases of low-grade B-cell lymphomas of MALT from different origin using α -satellite-probes specific for chromosomes 3, 7, 12 and 18.¹⁶ They found trisomy of chromosome 3 in 60%, compared to 16% in primary nodal low grade B-cell NHL. We could not reproduce the frequent finding of trisomy 3 in our series. Our only case with a trisomy 3 was a small cell lymphoma, which does correspond to other publications, mainly studying small cell lymphoma. However, the fact that the probe for chromosome 3 gave frequently rise to minor binding hybridisation spots, prompted us to wash this probe very stringently. Less stringent washing led to minor bindings, which were easily misinterpreted as positive signals. Using stringent washing and amplification of the signal with the CARD system led, however, to more easily evaluable signals.

As can be noticed from figure 6.1, cases showing less than 10% nuclei with three spots for chromosome 3, are concentrated at a higher percentage than the percentage of aberrant nuclei for the other chromosomes. This can be interpreted in different ways, first it is possible that the evaluation of *in situ* hybridisation spots for chromosome 3 is difficult because of minor binding hybridisation signals, second, it can represent true trisomic cases for chromosome 3. However, taking into account the technical problems encountered in the ISH for chromosome 3, it seems safer to use a high cut-off level.

The finding of trisomies for chromosome 7, 12 and 18 corresponds with those earlier mentioned in the literature, although we found these in lower frequencies too than reported in the literature. This may, however, be related to the use of

different cut-off levels in different studies (note figure 6.1).

Lymphomas are thought to be derived from one altered cell with a growth advantage. The finding of the same chromosomal aberrations in all cells of one tumour supports this idea. However, subpopulations can evolve because of genetic instability of the neoplastic cells. Late changes often represent the transformation from indolent to aggressive behaviour. In a recent study it was proposed that in a subset of gastric MALT lymphomas deletions of the p16 gene (located on chromosome 9p21) are acquired and may contribute to the transformation from a low grade to a high grade malignancy⁴⁰.

Our conclusions are hampered by the low number of cases, but may indicate that in regard of the fact that large cell cases show in part the same and, furthermore, more variant numerical chromosomal aberrations it is plausible that large cell lymphomas represent progression from small cell primary gastric lymphoma. Furthermore, we cannot reproduce the earlier reported frequency (60%) of small cell primary gastric lymphomas with high percentages of nuclei showing trisomy 3.

References

1. Speaks SL, Sanger WF, Linder J, Johnson DR, Armitage JO, Weisenburger D, Purtilo D. Chromosomal abnormalities in indolent lymphoma. *Cancer Genet Cytogenet* 1987;27:335-344.
2. Schouten HC, Sanger WG, Weisenburger DD, Anderson J, Armitage JO. Chromosomal abnormalities in untreated patients with non-Hodgkin's lymphoma: Associations with histology, clinical characteristics and treatment outcome. *Blood* 1990;75:1841-1847.
3. Levine EG, Arthur DC, Frizzera G, Peterson BA, Hurd DD, Bloomfield CD. There are differences in cytogenetic abnormalities among histologic subtypes of the non-Hodgkin's lymphomas. *Blood* 1985;66:1414-1422.
4. Johansson B, Mertens F, Mitelman F. Cytogenetic evolution patterns in non-Hodgkin's lymphoma. *Blood* 1995;86:3905-3914.
5. Hammond DW, Goepel JR, Aitken M, Hancock BW, Potter AM, Goyns MH. Cytogenetic analysis of a united kingdom series of non-Hodgkin's lymphomas. *Cancer Genet Cytogenet* 1992;61:31-38.
6. Whang-Peng J, Knutsen T, Jaffe ES, Steinberg SM, Raffeld M, Zhao WP, Duffy P, Condrón K, Yano T, Longo DL. Sequential analysis of 43 patients with non-Hodgkin's lymphoma: clinical correlations with cytogenetic, histologic, immunophenotyping, and molecular studies. *Blood* 1995;85:203-216.
7. Koduru PRK, Filippa DA, Richardson ME, Ihanwar SC, Chaganti ST, Koziner B, Clarkson BD, Lieberman PH, Chaganti RSK. Cytogenetic and histologic correlations in malignant lymphoma. *Blood* 1987;69:97-102.
8. Sreenan JJ, Tubbs RR. The influence of immunology and genetics on lymphoma classification: a historical perspective. *Cancer Invest* 1996;14:572-588.
9. Harris NL, Jaffe ES, Stein H, Banks PM, Chan JKC, Cleary ML, Delsol G, De Wolf-Peters C, Falini B, Gatter KC, Grogan TM, Isaacson PG, Knowles DM, Mason DY, Muller-Hermelink HK, Pileri SA, Piris MA, Ralfkiaer E, Warnke, RA. A revised European-American Classification of Lymphoid Neoplasms: A proposal from the international lymphoma study group. *Blood* 1994;84:1361-1392.
10. Harris NL. Principles of the revised European-American lymphoma classification (from the International Lymphoma Study Group). *Ann Oncol* 1997;8:S11-S16.
11. Poddighe PJ, Ramaekers FCS, Hopman AHN. Interphase cytogenetics of tumours. *J Pathol* 1992;166:215-224.
12. Hopman HNH, Voorter CEM, Ramaekers FCS. Detection of genomic changes in cancer by in situ hybridization. *Mol Biol Rep* 1994;19:31-44.
13. Anastasi J, LeBeau MM, Vardiman JW, Westbrook CA. Detection of numerical chromosomal abnormalities in neoplastic hematopoietic cells by in situ hybridization with a chromosome-specific probe. *Am J Pathol* 1990;136:131-139.

14. Lee W, Han K, Harris CP, Shim S, Kim S, Meisner LF. Use of FISH to detect chromosomal translocations and deletions. Analysis of chromosome rearrangement in synovial sarcoma cells from paraffin-embedded specimens. *Am J Pathol* 1993;143:15-19.
15. Isaacson P, Wright DH. Malignant lymphoma of mucosa-associated lymphoid tissue. *Cancer* 1983;52:1410-1416.
16. Wotherspoon AC, Finn TM, Isaacson PG. Trisomy 3 in low-grade B-cell lymphomas of mucosa-associated lymphoid tissue. *Blood* 1995;85:2000-2004.
17. Robledo M, Benitez J, Rivas C, Martinez-Castro P. Cytogenetic study of B-cell lymphoma of mucosa-associated lymphoid tissue. *Cancer Genet Cytogenet* 1992;62:208-209.
18. Isaacson PG, Spencer J. Malignant lymphoma of mucosa-associated lymphoid tissue. *Histopathology* 1987;11:445-462.
19. Schutte B, Reynders MJ, Bosman FT, Blijham GH. Flow cytometric determination of DNA ploidy level in nuclei isolated from paraffin-embedded tissue. *Cytometry* 1985;6:26-30.
20. Cooke H, Hindley J. Cloning of human satellite III DNA: different components are on different chromosomes. *Nucleic Acid Res* 1979;6:3177-3197.
21. Waye JS, Willard HF. Chromosome specificity of satellite DNAs: short- and long-range organization of a diverged dimeric subset of human alpha satellite from chromosome 3. *Chromosoma* 1989;97:475-478.
22. Waye J, England S, Willard H. Genomic organization of alpha satellite DNA on human chromosome 7: Evidence for two distinct alphoid domains on a single chromosome. *Mol Cell Biol* 1987;7:349-456.
23. Donlon T, Wyman A, Mulholland J, Banker D, Burns G, Latt S, Botstein D. Alpha satellite-like sequences at the centromere of chromosome 8. *Am J Hum Genet* 1986;39:A196.
24. Moyzis R, Albright K, Bartholdi M, Cram LS, Deaven LL, Hildebrand CE, Joste NE, Longmire JL, Meyne J, Schwarzscher-Robinson T. Human chromosome-specific repetitive DNA sequences: novel markers for genetic analysis. *Chromosoma* 1987;95:375-382.
25. Waye J, Creeper L, Willard H. Organization and evolution of alpha satellite DNA from human chromosome 11. *Chromosoma* 1987;95:182-188.
26. Looijenga L, Smit V, Wessels J. Localization and polymorphism of a chromosome 12-specific alpha satellite DNA sequence. *Cytogenet Cell Genet* 1990;54:216-218.
27. Higgins M, Wang H, Shtromas I, Haliotis T, Roder JC, Holden JJ, White BN. Organization of a repetitive human 1.8 kb KpnI sequence localized in the heterochromatin of chromosome 15. *Chromosoma* 1985;93:77-86.
28. Waye J, Willard H. Molecular analysis of a deletion polymorphism in alpha satellite of human chromosome 17: Evidence for homologues unequal crossing-over and subsequent fixation. *Nucleic Acid Res* 1986;14:6915-6927.

29. Devilee P, Cremer T, Slagboom P, Bakker E, Scholl HP, Hager HD, Stevenson AF, Cornelisse CJ, Pearson PL. Two subsets of human alphoid repetitive DNA show distinct preferential localization in the pericentromeric regions of chromosomes 13, 18 and 21. *Cytogenet Cell Genet* 1986;41:193-202.
30. Bobrow MN, Harris TD, Shaughnessy KJ, Litt GJ. Catalyzed reporter deposition, a novel method of signal amplification. Application to immunoassays. *J Immunol Method* 1989;125:279-285.
31. Bobrow MN, Litt GJ, Shaughnessy KJ, Mayer PC, Conlon J. The use of catalyzed reporter deposition as a means of signal amplification in a variety of formats. *J Immunol Method* 1992;150:145-149.
32. Hopman AHN, Ramaekers FCS, Raap AK, Beck JL, Devilee P, van der Ploeg M, Vooy's GP. In situ hybridization as a tool to study numerical chromosome aberrations in solid bladder cancer. *Histochemistry* 1989;89:307-316.
33. Chan JKC, Ng CS, Isaacson PG. Relationship between high-grade lymphoma and low-grade B-cell mucosa-associated lymphoid tissue lymphoma (MALToma) of the stomach. *Am J Pathol* 1990;136:1153-1163.
34. Montalban C, Manzanal A, Castrillo JM, Escribano L, Bellas C. Low grade gastric B-cell MALT lymphoma progressing into high grade lymphoma. Clonal identity of the two stages of the tumour, unusual bone involvement and leukaemic dissemination. *Histopathology* 1995;27:89-91.
35. Wotherspoon AC, Pan L, Diss TC, Isaacson PG. Cytogenetic study of B-cell lymphoma of mucosa-associated lymphoid tissue. *Cancer Genet Cytogenet* 1992;58:35-38.
36. Horsman D, Gascoyne R, Klasa R, Coupland R. t(11;18)(q21;q21.1): a recurring translocation in lymphomas of mucosa-associated lymphoid tissue (MALT) ? *Genes Chrom Cancer* 1992;4:183-187.
37. Clark HM, Jones DB, Wright DH. Cytogenetic and molecular studies of t(14;18) and t(14;19) in nodal and extranodal B-cell lymphoma. *J Pathol* 1992;166:129-137.
38. Whang-Peng J, Knutsen T, Jaffe E, Raffella M, Zhao WP, Duffey P, Longo DL. Cytogenetic study of two cases with lymphoma of mucosa-associated lymphoid tissue. *Cancer Genet Cytogenet* 1994;77:74-80.
39. Wotherspoon AC, Soosay GN, Diss TC, Isaacson PG. Low-grade primary B-cell lymphoma of the lung. An immunohistochemical, molecular, and cytogenetic study of a single case. *Am J Clin Pathol* 1990;94:655-660.
40. Neumeister P, Hoefler G, Beham-Schmid C, Schmidt H, Apfelbeck U, Schaidt H, Linkesch W, Sill H. Deletion analysis of the p16 tumor suppressor gene in gastrointestinal mucosa-associated lymphoid tissue lymphomas. *Gastroenterology* 1997;112:1871-1875.

Chapter 7

Retrospective survival analysis in gastric lymphoma reveals surgical therapy and MALT histology as favourable prognostic factors

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Summary

Background

Primary gastric lymphoma is a condition with a pathogenesis and biology different from primary nodal lymphoma. Its prognosis seems to be more favourable than nodal NHL. However, there is still no general agreement on the treatment of choice in primary gastric NHL.

Study design

The present population- based study retrospectively summarizes the presentation, histopathology, treatment and survival of 69 consecutive patients with primary gastric NHL. In addition to the clinical description of these cases, we tried to analyze the role of surgery in the treatment of gastric lymphoma, and to identify prognostic factors.

Materials and methods

Thirty-seven male and 32 female patients with a median age of 67 years were studied. They were classified as small cell MALT NHL (n=27), large cell MALT NHL (n=22) and large B-cell NHL (n=20).

Results

Abdominal pain, dyspepsia and weight loss were common complaints at presentation. The majority of patients presented in clinical stage I, without a significant difference in the three distinct groups. Forty-four patients underwent surgical therapy (17 of which in combination with chemotherapy and 5 with radiotherapy). Twenty-one patients received chemotherapy only. Overall five-year survival was 46%, whereas the projected ten-year survival was almost 37%.

Small cell lymphoma, MALT lymphoma, surgery, low stage (I, II) and younger age were associated with a longer survival, whereas the presence of p53 protein overexpression signified a poor prognosis. In a multivariate analysis, the presence of MALT and younger age were the only significant prognostic factors. To examine the role of therapy, only factors known at presentation were entered in the Cox regression. This procedure revealed surgery as the most important prognostic factor.

Conclusion

Gastric lymphoma with MALT component is associated with a better overall survival. Furthermore, within the limitations of this retrospective analysis, surgically treated patients had a more favourable prognosis.

Introduction

Fifty percent of non-Hodgkin's lymphoma has an extranodal component. Of these extranodal localisations primary gastric lymphoma is the most frequent. Recently, much attention is paid to this relatively uncommon condition, mainly because of its interesting pathogenesis. These lymphomas originate in the stomach, which normally contains no organized lymphoid tissue. However, mucosa-associated lymphoid tissue (MALT) can develop in the stomach in response to a chronic *Helicobacter pylori* gastritis^{1,2}. It is yet unclear, which factors are responsible for the development of small cell MALT lymphoma and the possible progression to large cell lymphoma. Whereas small cell lymphomas almost always show the characteristic features of MALT (lymphoepithelial lesions), large cell lymphomas do not. Therefore, if a small cell MALT component is not present in biopsy or resection specimens the origin of such large cell lymphomas often remains unclear. As is clear from the above, the (histo-)pathological definition of MALT lymphoma has gained a lot of clarity, despite the relatively short history of the concept of MALT lymphoma. The introduction of the MALT concept for clinical use has been somewhat delayed, mainly because of its indistinct relevance for the clinical course. Moreover, only recently primary gastric lymphomas are classified as low and high grade MALT lymphoma³, whereas before, classifications designed for nodal lymphoma, like the Kiel⁴ and Working Formulation⁵ were used. The use of these different classification systems (and staging systems) made the interpretation and comparability of the clinical results very difficult. For these reasons, and the rarity of the disease, there is still no general agreement on prognostic factors and therapy.

In this study we intended to study small cell MALT lymphomas and large cell gastric lymphomas with and without small cell MALT features for differences in their clinical presentation, and survival. Furthermore, part of these cases were studied earlier for cell turnover indices in which it was shown that these indices correlated well with histology, the large cell lymphomas having higher proliferative and apoptotic indices when compared to the small cell cases⁶. In the present population-based study these data are correlated to clinical outcome.

Materials and methods

Patients

In this series, 69 patients with primary gastric lymphoma were evaluated retrospectively. Patients were selected by means of the registrations of the Limburg Working Party on Lymphoma, a panel of pathologists monthly discussing the

histology of all new diagnosed lymphomas in the province of Limburg (approximately 1 miljoen inhabitants). From this registry, all patients presenting with lymphoma in the stomach between 1986 and 1994 were selected (n=86) and clinical data and tissue blocks of these cases were obtained. After screening of these clinical data and revision of the material, cases with secondary involvement of the gastrointestinal tract (patients with disseminated disease were included if their predominant lesion was located in the stomach) and incorrect diagnosed cases were excluded (n=9). Cases without adequate clinical follow-up (n=5) were excluded and, furthermore, T-cell lymphomas were excluded (n=3).

Classification

All cases were reviewed and classified according to the classification proposed at the fifth international conference on malignant lymphoma³. Cases were classified as large cell lymphoma when they contained sheets of large blastic cells. Large cell lymphomas were considered to be of MALT origin when they displayed a small cell component showing MALT features in addition to the large cell histology^{7,8}. However, if no such small component could be detected, they were classified as diffuse large cell lymphoma, non-MALT.

Immunohistochemistry

In 45 cases data on the proliferative and apoptotic indices, studied using the mib-1 antibody and the TUNEL assay respectively, were also available. Furthermore, the expression of two gene-products involved in the regulation of apoptosis, the p53 and bcl-2 proteins was known. These data have been published in a previous study⁶. In the present study, these data were correlated to the clinical outcome of the patients.

Clinical data

Clinical information was gathered from clinical and outpatient files. Data on presentation, history, staging therapy and outcome were recorded. Clinical staging was performed according to the modified Ann Arbor classification⁹. Patients were followed from diagnosis up till December 1996, till the date of last follow-up or till death. The median follow-up of patients alive at last follow-up was 2221 days ranging from 267-3344 days.

Statistical analysis

The overall survival was calculated from the date of diagnosis until the date of last follow-up or death. Differences between small and large cell lymphoma as described in table 7.1 were compared using chi-square and Mann-Whitney U tests. Survival analysis was performed by Kaplan-Meier estimations¹⁰ and differences

between subgroups were analyzed using the log-rank test. A Cox's multivariate analysis was performed to determine which variables were of independent value in predicting outcome¹¹. To determine prognostic factors, all variables showing significant effect on survival in univariate analysis were included in the multivariate analysis. Analysis of the role of surgery was performed by including significant variables known at presentation.

Table 7.1 Presenting symptoms and signs in primary gastric small and large cell NHL.

	small cell	large cell	P
Age	26-83, 65	35-91, 67	ns
Sex M/F	14/13	23/19	ns
Presentation, n*	26	41	
Pain	19 (73.1)	31 (75.6)	ns
Dyspepsia	11 (42.3)	16 (39.0)	
Weight loss	8 (30.8)	14 (34.1)	
Nausea	10 (38.5)	11 (26.9)	
Anorexia	6 (23.0)	11 (26.9)	
Blood loss	7 (26.9)	7 (17.1)	
Vomiting	6 (23.1)	5 (12.1)	
Diarrhoea	3 (11.5)	3 (7.3)	
Fever	1 (3.8)	3 (7.3)	
Obstruction	-	3 (7.3)	
Transpiration	2 (7.7)	1 (2.4)	
Perforation	1 (3.8)	1 (2.4)	
Physical Examination, n*	25	40	ns
Hepatomegaly	4 (16.0)	7 (17.5)	
Splenomegaly	2 (8.0)	3 (7.5)	
Lymphadenopathy	1 (4.0)	1 (10)	
Stage			ns
I	19 (70.4)	24 (57.1)	
II1	-	8 (19.0)	
II2	1 (3.7)		
III	2 (7.4)	3 (7.1)	
IV	3 (11.1)	5 (11.9)	
unknown	1 (3.7)	2 (4.8)	
Medical History, n*	26	41	
Peptic ulcer disease	9 (34.6)	13 (31.7)	

Results

Clinical features

Thirty-seven patients were male and 32 patients were female with an age range of 26 to 91 years (median age: 67 years). Twenty-seven cases were classified as small cell MALT lymphoma, 22 cases as large cell lymphomas with a small cell MALT origin and 20 cases large cell non-MALT. Table 7.1 shows some characteristics on age, sex, presentation, physical examination and stage in small and large cell lymphomas. The most common complaint at presentation was abdominal pain, in

almost 75% of the patients, followed by dyspepsia (40%) and weight loss (30%). No significant differences between small and large cell lymphomas were found for age and sex distribution and other parameters tested. Physical examination showed hepatosplenomegaly in 16% and splenomegaly in 8%, without differences for small or large cell lymphoma. Thirty percent of the patients had a history of peptic ulcer disease, or presented with peptic ulcer symptoms which at histological examination proved malignant. Table 7.2 shows the clinical course of the individual cases (smM = small cell MALT, la = large cell, laM = large cell MALT, C = chemotherapy, R = radiotherapy, S = surgery, Y = recurrent disease, + = patient still alive at last follow-up).

Therapy

Seven patients received no treatment, four of these were considered too frail for therapy, one patient died before intervention and in two patients were not treated for unknown reasons. Forty-four patients were treated with surgery, in most cases a partial gastrectomy. Seventeen of these patients received additional chemotherapy and five patients additional radiotherapy. Nineteen patients underwent chemotherapy only. Twenty-five of the thirty-six patients treated with chemotherapy were subjected to an adriamycin-containing regimen. Twenty-seven patients with large cell lymphoma (total 42 patients) got chemotherapy, 20 of which an adriamycin-containing regimen.

Outcome

Eight patients had recurrent disease, three in the regional nodes, three locally in the stomach and two in the lungs. Thirty-seven patients died during the follow-up period, 12 of which of non-lymphoma related causes. Three of these patients died of other malignancies (carcinoma of lung, colon and rectum respectively). Fifteen patients died of direct lymphoma related causes, five of which as a result of therapy (three because of complicated surgery, two because of sepsis due to chemotherapy induced cytopenia). The remaining ten patients died of progressive disease.

Univariate survival analysis

Patients with small cell primary gastric lymphoma had a significantly better prognosis compared to large cell cases (log-rank test, $p=0.0437$), and patients with small and large MALT NHL had a better prognosis than non-MALT NHL ($p=0.0005$). Patients younger than the median age of 67 also had a more favourable prognosis ($p=0.028$). Stage I and II disease was associated with a better outcome compared to stage III and IV disease (log-rank test, $p=0.0352$). Furthermore, patients treated surgically had a significantly better survival

Table 7.2 Patient characteristics.

no	age/sex	Histol/stage	Therapy	Rec.	survival	Cause of death
1			S	-	20	gastric bleeding
2	52/♀	smM/I	C	-	3344+	
3	70/♂	smM/IIIa	S/C	-	3250+	
4	56/♂	laM/I	-	-	18	gastric bleeding
5	86/♂	laM/IV	C	-	1669	DWD
6	75/♀	la/I	S/C	-	3114+	
7	51/♀	laM/I	S	-	2963+	
8	31/♀	smM/I	S	Y	282	progressive disease
9	50/♂	smM/I	S/C	-	2467+	
10	35/♂	laM/I	S	-	2974	DWD lungcancer
11	75/♂	laM/I	S	Y	329	ileus (recurrent disease)
12	66/♂	la/IIIe	S/C	-	2954+	
13	59/♂	smM/I	S	-	267+	
14	26/♂	smM/I	-	-	2252+	
15	67/♂	smM	S/C	-	58	sepsis due to cytopenia (chemotherapy)
16	69/♀	laM/I	S/C	-	2631+	
17	59/♂	laM/IIIe	C	-	475	progressive disease
18	59/♀	la/III	C	-	216	progressive disease
19	50/♂	la/IIIse	S	-	3035+	
20	70/♂	smM/	S/C	-	2485+	
21	52/♂	laM/III	S	-	2300+	
22	83/♀	T/I	S/R	-	2804+	
23	42/♀	smM/I	C	Y	422	gastric bleeding (recurrent disease)
24	71/♂	la/IIIa	S/C	-	2235	
25	75/♀	laM/I	S	-	2314+	
26	64/♂	smM/I	C	-	168	sepsis, cytopenia
27	60/♀	la/	-	-	274	DWD myocardial infarction
28	83/♀	smM/	S/C	-	2221+	
29	43/♀	smM/I	-	-	12	DWD Heart failure
30	85/♀	la/I	S	-	73	Complicated surgery
31	77/♀	laM/I	S/C	-	2707+	
32	70/♀	laM/III	C	-	1704	DWD Heart Failure
33	67/♀	la/IV	C	-	1520	DWD Shock due to other surgery
34	78/♂	smM/I	S	-	1911+	
35	57/♀	la/I	C/R	-	293	Progression, vena cava inf. syndr.
36	71/♀	smM/IV	S	-	68	Complicated surgery
37	72/♂	laM/I	S/R	Y	1091	Respiratory failure (lung metastases)
38	70/♂	smM/I	C	-	1747+	
39	75/♀	T/IIIe	S/R	-	201	Ruptured spleen > shock
40	67/♀	la/I	C	Y	1694	progressive disease
41	59/♂	T/IV	-	-	384	DWD Resp. failure (urosepsis)
42	79/♂	smM/I	S/C	-	2277+	
43	46/♂	la/I	C	-	1852	DWD myocardial infarction
44	70/♀	smM/IIIe	C	-	370	DWD
45	73/♂	smM/IIIse	S	-	1728+	
46	62/♂	smM/I	S	Y	1526+	
47	50/♀	smM/IIIe	C/R	-	2266+	
48	56/♂	laM/I	S	-	2105+	
49	51/♀	smM/I	S	-	2207+	
50	77/♀	laM/I	-	-	20	Progressive disease
51	81/♂	la/I	C	-	1043	DWD
52	70/♂	laM/IV	C	-	354	Respiratory failure
53	82/♀	smM/IV	S	-	1319+	
54	69/♀	smM/I	S/C	-	1394+	
55	70/♀	laM/III	S	-	1994+	
56	51/♀	smM/I	S/C	-	1071+	
57	58/♂	laM/I	C	-	301	Livermetastasis rectumcarcinoma
58	65/♂	la/IV	S	Y	673	Ileus (recurrent disease)
59	73/♂	la/I	S	-	45	Gastric bleeding
60	64/♂	laM/I	S	-	1984+	
61	74/♀	laM/I	C	Y	370	Progressive disease
62	61/♂	laM/IIIa	C	-	238	Lung metastasis
63	67/♀	la/I	S	-	620	Colon carcinoma
64	50/♂	laM/I	-	-	28	progressive disease
65	56/♀	smM/I	S	-	616+	
66	52/♀	la/I	S/C	-	677+	
67	59/♂	la/I	S/C	-	213	pneum. Carinii (lymphocytopenia)
68	76/♂	smM/I	S	-	542+	
69	74/♂	la/I	S/C	-	22	Cachexia, progressive disease
70	65/♂	smM/IV	C	-	472+	
71	62/♂	-/IIIe	C	-	466+	
72	69/♂	la/III	S/C	-	91	

($p=0.0006$). Proliferative and apoptotic indices did not show any prognostic value. The p53-positive cases demonstrated a significantly worse survival ($p<0.001$), but only 3 cases were p53 positive.

Multivariate survival analysis

Variables significant in univariate analysis were entered into the multivariate analysis with exception of p53 overexpression because of the very low frequency of positivity (Table 7.3). The presence of MALT histology and young age came out as independent prognostic variables (Figure 7.1).

To analyze whether surgery was an important variable in predicting outcome, the presence of MALT was excluded, because in most cases presence or absence of a small cell MALT component could only be established in resection specimens. When entering surgery, small or large cell histology, age and stage into the multivariate analysis surgery appeared to be the only independent prognostic variable (Table 7.4).

Table 7.3 Results of univariate and multivariate analysis and RR.

Variable	Univariate analysis	Multivariate analysis	RR
small vs large cell	$p = 0.0437$	$p = 0.7222$	1.1920
p53	$p = 0.0001$	excluded	
surgery/no surgery	$p = 0.0006$	$p = 0.1073$	0.5373
stage I,II vs III, IV	$p = 0.0352$	$p = 0.7763$	1.1382
age $>/<$ median age	$p = 0.028$	$p = 0.0069$	2.7040
MALT features	$p = 0.0005$	$p = 0.0003$	0.2792

Figure 7.1 Kaplan-Meier curves of combinations of age and MALT-histology as prognostic variables.

Table 7.4 Results of multivariate analysis with exclusion of variables not known at presentation.

Variable	Multivariate analysis	RR
Stage I, II vs III, IV	p=0.8396	1.0970
Surgery/no surgery	p=0.0060	0.3693
age >/< median	p=0.0502	2.0990
small vs large cell	p=0.0511	2.1078

Additional analyses in large cell (MALT and non-MALT) lymphoma cases

Large cell lymphomas with a MALT component had a significantly better survival than those in which no such small cell component could be detected (log rank test, $p=0.0167$). This was also the case for surgery ($p=0.0064$), stage ($p=0.0146$) and younger age ($p=0.0427$). In addition, patients treated with both surgery and chemotherapy had a better outcome than those who had surgery only ($p=0.0472$). Correlation of survival data with proliferation and apoptosis, showed a borderline significant role for apoptosis, lymphomas with a high apoptotic index showed better outcome ($p=0.0595$).

Discussion

The most striking finding from this study is that multivariate survival analysis revealed MALT histology as an independent prognostic factor in gastric lymphomas. At a first glance, this could be explained by our observation that lymphomas with a MALT histology were both small and large cell lymphomas, whereas all non-MALT lymphomas belonged to the large cell lymphoma group. However, this explanation flaws, because analysis in large cell lymphomas the MALT subgroup also showed a significantly better survival. In general, this finding would nicely fit into the hypothesis that MALT lymphoma has a better survival compared to nodal lymphoma because of its unique features. It should be minded, however, that the exact nature of the large cell lymphomas without a small cell MALT component could be questionable: either a small cell component has not been included for diagnostic analysis, or they may have been MALT lymphomas but completely overgrown during progression by the large cell component. Despite these uncertainties, it is surprising that large cell lymphomas with a MALT component have a better clinical course compared to diffuse large cell lymphomas.

In the earliest reports on MALT lymphoma, the homing pattern of normal mucosal lymphocytes was thought to be reflected in the late dissemination of these lymphomas⁷. According to the REAL classification MALT lymphomas are considered to be of marginal zone origin¹². Marginal zone lymphocytes probably do not recirculate at all, which might also explain the longer localized stage^{13,14}. In the present study, 67.3% of MALT lymphomas presented in stage I, whereas 50% of non-MALT lymphomas presented in stage I.

Another unexpected finding in this study is the role of surgery in the survival of gastric lymphoma. In recent years, stomach-preserving therapy has gained more and more attention. Salles and colleagues in 1991 found a similar survival in high grade gastric lymphoma patients with complete, incomplete or no surgical therapy before chemotherapy and they concluded that chemotherapy is the elective treatment in this group¹⁵. Muller et al. however, noted a trend for improved survival in patients treated with surgery¹⁶. They reasoned that this finding might reflect differences in age and stage at presentation between the two groups. Data from the present study reveal surgery as the only significant prognostic factor among variables known at presentation. In this study the surgery-treated patients were significantly younger (Mann-Whitney U-test, $p=0.0013$) with a mean age of 60 versus 71 years in the patients without surgical treatment. Also, all but one of the surgically treated patients presented in stage I or II. The role of surgery appears to be strongly interwoven with the presence of MALT features. There may be two reasons for this association: first, the presence of gastrectomy specimens raises the chance of detecting a small cell MALT component, because more material is present. Second, selection of patients with MALT lymphoma for surgery may have been associated with a lower stage of disease at presentation, so that a bias is induced. Exactly how these variables are associated cannot be retrieved from the present study, but should prompt to further investigation in a prospective setting. Median ages in patients with small and large cell lymphomas did not differ significantly with a slightly higher age in patients presenting with large cell lymphomas. This, and the higher frequency of primary gastric lymphoma in males is confirmed in other studies^{17,18}. Not very surprising, given the association of *Helicobacter pylori* with both diseases and the frequent presentation of gastric lymphoma as an ulcerative condition^{19,20}, was the finding that one third of the patients had a history of peptic ulcer disease or presented with peptic ulcer disease. However, this supports the idea that extensive biopsy sampling is necessary in patients with gastritis or peptic ulcer disease. Like in other series, pain, dyspepsia and weight loss were common symptoms at presentation²¹⁻²³. As no differences in presentation of small and large cell gastric lymphoma were observed, the clinical presentation cannot be used to predict grade of the lymphoma, a conclusion which can also be drawn from other recent studies²⁴. A high percentage of patients (64%) presented with stage I disease, a similar percentage as that described by Hoshida

e.a. (66%) who also used the MALT concept for classification²⁵. However, a finding not shared by the same authors is presentation in stage IV in about 11% of (both small and large cell) lymphoma patients in our series. This finding may explain in part the lower 5-year overall survival in this series compared to the series of Hoshida. Furthermore, they describe a series of patients selected on gastrectomy, and as turned out in the present study, patients treated with surgery had a better overall survival.

The present study intended to describe the clinical features and course of a retrospective series of gastric lymphoma, revised according to the current MALT classification. Furthermore, proliferative and apoptotic indices studied before in combination with the expression of p53 and bcl-2 were related to the clinical data. In conclusion, clinical signs and symptoms in gastric lymphoma are aspecific, and resemble other gastric diseases. Furthermore, they cannot be used to differentiate between small and large cell lymphoma. Proliferation and apoptosis did not play a significant role in the outcome of the patients. The presence of MALT features, younger age and surgical therapy, however, came out as very significant factors influencing survival. Given the retrospective nature of this study these intriguing findings deserve further evaluation.

References

1. Zaitoun AM. The prevalence of lymphoid follicles in *Helicobacter pylori* associated gastritis in patients with ulcers and non-ulcer dyspepsia. *J Clin Pathol* 1995;48:325-329.
2. Eidt S, Stolte M. Prevalence of lymphoid follicles and aggregates in *Helicobacter pylori* gastritis in antral and body mucosa. *J Clin Pathol* 1993;46:832-835.
3. Rohatiner A, D'Amore F, Coiffier B, Crowther D, Gospodarowicz M, Isaacson P, Lister TA, Norton A, Salem P, Shipp M, Somers R. Report on a workshop convened to discuss the pathological and staging classifications of gastrointestinal tract lymphoma. *Ann Oncol* 1994;5:397-400.
4. Stansfeld AG, Diebold J, Kapanci Y, Kelenyi G, Lennert K, Mioduszevska O, Noel H, Rilke F, Sundstrom C, Van Unnik JAM, Wright D. Updated Kiel classification for lymphomas. *Lancet* 1988;6:292-293.
5. Rosenberg SA. National Cancer Institute sponsored study of classifications of non-Hodgkin's lymphomas. Summary and description of a Working Formulation for clinical usage. *Cancer* 1982;49:2112-2135.
6. Gisbertz IAM, Schouten HC, Bot FJ, Arends J-W. Proliferation and apoptosis in primary gastric B-cell non-Hodgkin's lymphoma. *Histopathology* 1997;30:152-159.
7. Isaacson P, Wright DH. Malignant lymphoma of mucosa-associated lymphoid tissue. *Cancer* 1983;52:1410-1416.
8. Isaacson PG, Spencer J. Malignant lymphoma of mucosa-associated lymphoid tissue. *Histopathology* 1987;11:445-462.
9. Musshoff K. Klinische Stadieneinteilung der Nicht-Hodgkin-Lymphome. *Strahlentherapie* 1977;153:218-222.
10. Kaplan EL, Meier P. Non parametric estimation from incomplete observations. *J Am Stat Assoc* 1958;53:457-81.
11. Cox DR. Regression models and life tables. *J Stat Soc* 1972;34:187-220.
12. Harris NL, Jaffe ES, Stein H, Banks PM, Chan JKL, Cleary ML, Delsol G, De Wolf-Peters C, Falini B, Gatter KC, Grogan TM, Isaacson PG, Knowles DM, Mason DY, Muller-Hermelink HK, Pileri SA, Piris MA, Ralfkiaer E, Warnke RA. A revised European-American Classification of Lymphoid Neoplasms: A proposal from the international lymphoma study group. *Blood* 1994;84:1361-1392.
13. Spencer J, Finn T, Pulford KAF, Mason DY, Isaacson PG. The human gut contains a novel population of B lymphocytes which resemble marginal zone cells. *Clin Exp Immunol* 1985;62:607-612.
14. MacLennan ICM, Gray D, Kumararatne DS, Bazin H. The lymphocytes of splenic marginal zones: a distinct B-cell lineage. *Immunol Today* 1982;3:305-307.
15. Salles G, Herbrecht R, Tilly H, Berger F, Brousse N, Gisselbrecht C. Aggressive primary gastrointestinal lymphomas: review of 91 patients treated with the LNH-84 regimen. A study of the Groupe d'Etude des Lymphomes Agressifs. *Am J Med* 1991;90:77-84.
16. Muller AF, Maloney A, Jenkins D, Dowling F, Smith P, Bessel EM, Toghill PF. Primary gastric lymphoma in clinical practise. 1973-1992. *Gut* 1995;36:679-683.

17. Azab MB, Henry-Amar M, Rougier P, Bognel C, Theodore C, Caude P, Lasser P, Cosset JM, Caillou B, Droz JP, Hayat M. Prognostic factors in primary gastro-intestinal non-Hodgkin's lymphoma. *Cancer* 1989;64:1208-1217.
18. Castrillo JM, Montalban C, Obeso G, Piris MA, Rivas MC. Gastric B-cell mucosa-associated lymphoid tissue lymphoma: a clinicopathological study in 56 patients. *Gut* 1992;33:1307-1311.
19. Rautelin H, Kosunen TU. *Helicobacter pylori* and associated gastroduodenal disease. *APMIS* 1991;99:677-695.
20. Moss S, Calam J. *Helicobacter pylori* and peptic ulcers: the present position. *Gut* 1992;33:289-292.
21. Tondini C, Giardini R, Bozzetti F, Valagussa P, Santoro A, Bertulli R, Balzarotti M, Rocca A, Lombardi F, Ferreri AJM, Bonadonna G. Combined modality treatment for primary gastrointestinal non-Hodgkin's lymphoma: The Milan Cancer Institute Experience. *Ann Oncol* 1993;4:831-837.
22. Amer MH, El-Akkad S. Gastrointestinal lymphoma in adults: clinical features and management of 300 cases. *Gastroenterology* 1994;106:846-858.
23. Taal BG, Burgers JMV, VanHeerde P, Hart AAM, Somers R. The clinical spectrum and treatment of primary non-Hodgkin's lymphoma of the stomach. *Ann Oncol* 1993;4:839-846.
24. Taal BG, Boot H, VanHeerde P, DeJong D, Hart AAM, Burgers JMV. Primary non-Hodgkin lymphoma of the stomach: endoscopic pattern and prognosis in low versus high grade malignancy in relation to the MALT concept. *Gut* 1996;39:556-561.
25. Hoshida Y, Kusakabe H, Furukawa T, Miwa H, Ishiguro S, Aozasa K. Reassessment of gastric lymphoma in the light of mucosa-associated lymphoid tissue lymphoma: Analysis of 53 patients. *Cancer* 1992;80:1151-1159.

Chapter 8

Cell turnover parameters in primary intestinal non-Hodgkin's lymphoma related to small and large cell varieties

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Summary

Background

In contrast to primary gastric; primary intestinal lymphoma is an uncommon condition with a worse outcome, perhaps due to differences in pathogenesis. In this study we analyzed the role of proliferation and apoptosis in the pathogenesis of intestinal lymphoma.

Methods

Fifty-one cases of intestinal NHL (10 small B-cell MALT NHL, 12 large B-cell MALT NHL, 18 large B-cell NHL, 2 small T-cell NHL, 7 large T-cell NHL, 2 mantle cell NHL) were studied for the immunohistochemical expression of Mib-1 and the TUNEL assay respectively, together with the expression of two regulatory gene-products involved in apoptosis, bcl-2 and p53.

Results

We found a median proliferation index (PI) of 37.3% and a median apoptotic index (AI) of 1.10%. The respective values of PI and AI in small B-cell MALT lymphoma were 5.8%/0.06%, large B-cell MALT lymphoma: 52.8%/0.24%, large B-cell lymphoma: 58.85%/1.36%, mantle cell lymphoma: 30.9%/1.93%, small T-cell lymphoma: 18.13%/1.25% and large T-cell lymphoma: 43.4%/1.93%. Analysing B-cell NHL only (mantle cell NHL excluded) proliferative and apoptotic indices turned out to be positively correlated: 0.563, $p < 0.001$. Furthermore, high bcl-2 expression was inversely correlated with both PI and AI. Eight cases showed P53 expression, 1 small cell lymphoma and 7 large cell lymphomas.

Conclusions

In conclusion, small cell lymphomas showed low AI and PI, whereas large cell lymphomas demonstrated high AI and PI. Apoptosis and proliferation were positively correlated and a higher expression of bcl-2 was associated with lower rates of apoptosis.

Introduction

Gastrointestinal lymphoma is the most common form of extranodal lymphoma and can be divided according to localisation in the gastrointestinal tract into primary gastric lymphoma accounting for the majority of primary gastrointestinal lymphoma and primary intestinal lymphoma, comprising one-third of the cases. In the latter group, the small intestine is involved almost twice as often as the colon^{1,2}, but these figures are dependent on geographical variation. Considering primary lymphoma of the small intestine among other neoplasms at that site, lymphoma is the third most common tumour after adenocarcinomas and carcinoid tumours³. Colorectal lymphoma accounts for only 11% out of the three percent of colon neoplasms other than adenocarcinomas, but has a poor prognosis^{4,5}. In the United States the incidence of intestinal lymphoma is rising, in particular among immunocompromised patients, like those suffering from AIDS or those who have received immunosuppression, for example organ transplant recipients^{6,7}.

Because of its low incidence, intestinal lymphoma is often diagnosed at a late stage, a situation, which may aggravate the prognosis. Still little is known about the pathogenesis of this disease. We and others have previously investigated the role of cell proliferation and cell apoptosis in primary gastric lymphoma^{8,9} and found a significant difference in the regulation of both proliferation and apoptosis in small and large cell primary gastric lymphoma. To our best knowledge no such studies have so far been performed on intestinal lymphoma. Therefore, we intended to try and shed more light on the pathogenesis of intestinal lymphoma. We studied proliferation (assessed with the mib-1 antibody) and apoptosis (using the terminal deoxynucleotidyl transferase (TdT)-mediated digoxigenin-dUTP nick end labelling (TUNEL) assay^{10,11} in 42 cases. Furthermore, the expression of two regulatory proteins in the process of apoptosis was studied using immunohistochemistry: the bcl-2 gene product¹²⁻¹⁴, and the p53 protein¹⁵⁻¹⁷.

Materials and Methods

All cases presenting with non-Hodgkin's lymphoma located in the intestines - distal to the pylorus down to the anal canal - were selected from the files of the pathology departments of nine hospitals in the province of Limburg (The Netherlands). The formalin-fixed tissue blocks were retrieved and 2 μ m sections were cut and stained routinely with Haematoxylin-Eosin (HE) to review the histology. Only cases showing infiltration of the mucosa of the intestines were included, primary mesenteric lymphomas were excluded. Furthermore, Burkitt's lymphomas were excluded, because of their different pathogenesis and since this disease is most common in children¹⁸. Clinical data were retrieved to establish that

the intestines were indeed the primary site, i.e. the intestines had to be the only or the most massively affected organ and no former or concurrent nodal localisation of non-Hodgkin's lymphoma should be present. According to these criteria, 51 cases of primary intestinal lymphoma could be included, with sufficient material to perform analyses. Twenty-two cases were located in the small intestine, 13 in the colon, 7 ileocecal, 6 in the rectum, 2 in the appendix and 1 case was found in multiple sites in the intestinal tract. Haematoxylin and Eosin stained sections were reviewed by two pathologists (JWA, FJB) and were classified according to the classification recommended for gastrointestinal lymphomas by Rohatiner et al.¹⁹. MALT lymphoma was diagnosed as described by Isaacson²⁰⁻²²: the presence of reactive B-cell follicles, surrounded by the tumour cell infiltrate consisting of centrocyte like cells, selectively invading epithelial structures to form the characteristic lympho-epithelial lesions. Large cell lymphomas of MALT were diagnosed when the tumour contained sheets of large blastic cells, as well as a small cell component in the tumour displaying the typical features of MALT. Furthermore, a semiquantitative analysis of the number of diffusely intermingled blasts was performed, according to a recently described classification for gastric lymphoma²³. In addition, the B- or T-cell phenotype of the tumour was established. No cases with immunoproliferative small intestinal disease (IPSID) or enteropathy-associated T-cell lymphoma (EATL) were diagnosed. The final series consisted of 10 small cell MALT lymphomas, 12 large cell MALT lymphomas, 18 large B-cell lymphomas without a detectable small cell MALT component, 2 small T-cell lymphomas, 7 large T-cell lymphomas and 2 mantle cell lymphomas. Data from B-cell cases, T-cell cases and mantle cell lymphomas were analyzed separately. Most analyses were performed on B-cell lymphomas, because the number of T-cell and mantle cell lymphomas was too low. Therefore, if not otherwise indicated, the data in results and discussion concern B-cell lymphomas.

For immunohistochemistry serial sections of 2 μm were cut from routinely processed formalin-fixed paraffin-embedded tissues and were mounted onto 3-Amino-Propyl-triethoxy-Silane (APS) coated slides. The sections were deparaffinized in xylene and rehydrated in graded ethanols. To undo the masking effect of formalin fixation, all sections were placed in a boiling citrate buffer (2.1 g citric acid in 1 l of distilled H_2O ; pH to 6.0 with 5M NaOH) for 2 times 5 minutes in a microwave oven at 750 Watt. They were left to cool in the buffer for another hour. After washing in phosphate buffered saline (PBS), endogenous peroxidase was blocked with 0.3% H_2O_2 in methanol for 30 minutes. To block aspecific binding sites the slides were covered with 5% bovine serum albumin (BSA) in PBS. After washing in PBS the primary antibodies were applied: DO-7 (mouse monoclonal antibody recognizing an epitope in the N-terminus of the wild-type and mutant form of the human p53 protein, DAKO, ITK diagnostics, Uithoorn, The Netherlands) diluted 1:500, incubated for two hours at room temperature, Mib-1 (a

monoclonal mouse anti-human antibody which reacts to a nuclear antigen present in all phases of the cell cycle except for the G₀ phase; Dianova, Hamburg, Germany) diluted 1:100, incubated for one hour at roomtemperature and the mouse anti-human bcl-2 oncoprotein (clone 124, DAKO) diluted 1:100, incubated for two hours at roomtemperature. Negative controls were performed by omitting the primary antibody and as positive controls for bcl-2, mib-1 and p53 a follicular lymphoma, a reactive lymph node and a p53 positive breast carcinoma were used, respectively. After washing in PBS, primary antibodies were detected by incubation with biotinylated anti-mouse antibody (Vectastain Elite Kit, Vector, Burlingame, CA, USA) for 30 minutes at room temperature. After washing peroxidized avidin-biotin complex (Vectastain Elite Kit) was applied for 30 minutes at room temperature. Visualisation was achieved with 3,3'-diaminobenzidine-tetrahydrochloride (DAB) in a Tris-HCl buffer. The slides were counterstained lightly with haematoxylin and after dehydration were mounted with entellan.

For the in situ detection of apoptotic cells the terminal deoxynucleotidyl transferase (TdT)-mediated digoxigenin-dUTP nick end labelling (TUNEL) was used. Sections of 2 μ m were deparaffinized and rehydrated. Endogenous peroxidase was blocked as described above. Sections were pretreated with 20 μ g/ml of protein digesting enzyme (ONCOR, Sanbio, Uden, The Netherlands) for 15 minutes at room temperature. After washing in 4 changes of aquadest, equilibration buffer (Apoptag Kit, Oncor, Gaithersburg, Maryland, USA) was applied for 10 minutes at roomtemperature, followed by the application of 10 TI working strenght TdT enzyme (a mixture of 7.6 TI reaction buffer and 3.2 TI TdT enzyme, Apoptag Kit) per cm² tissue section for one hour at 37° Celsius in a humidified chamber and covered with a coverslip. The reaction was terminated in preheated Stop/Wash Buffer (Apoptag Kit) for 30 minutes at 37° Celsius. After washing in PBS, the digoxigenin-labelled dUTP polymer was detected by anti-digoxigenin-peroxidase (ApoptagKit) for 30 minutes at room temperature. After washing, visualisation was performed with DAB. The colour reaction was followed microscopically and the reaction was terminated when adequate staining was achieved. Sections were counterstained lightly with haematoxylin and after dehydration were mounted with entellan. A rat thymus was taken into the reaction as a positive control, and the TdT enzyme in the working strength TdT was replaced by aquadest as a negative control.

For the evaluation of mib-1, p53 and TUNEL, light microscopical evaluation was performed at a magnification of 400 times, with the aid of a counting grid. In all evaluations fields with the highest concentration of positive cells were examined. The positive nuclei in mib-1 staining were counted in three fields to a total of at least 500 neoplastic cells. From these observations the mean relative frequency (PI, %) was calculated. In the case of p53 staining, only tumours that contained more than 30% positive nuclei were regarded as bearing mutations in the p53 gene.

Lower percentages of p53-positive nuclei were taken to express the wild-type p53^{24,25}. TUNEL-positive nuclei were counted in 10 microscopical fields to a total of 2000 till 6000 tumour cells. The Apoptotic Index (AI) was determined by the ratio of TUNEL-positive neoplastic cells/total number of neoplastic cells. Bcl-2 staining revealed three staining patterns: high, intermediate and negative in which we noticed 1) high: almost all cells expressing bcl-2; 2) intermediate: bcl-2 positive (less than 60%) and negative tumour cells admixed in the same area; and 3) negative staining of almost all tumour cells (less than 5% positive). Statistical analysis was performed with the use of SPSS 6.1. In particular the Mann-Whitney U test, the Kruskal-Wallis 1-Way Anova and the Spearman correlation coefficient for non-parametric variables were used. A p-value of less than 0.05 was considered significant.

Table 8.1 Median proliferative index (PI) and apoptotic index (AI) in percentages in different histologic entities of primary intestinal non-Hodgkin's lymphoma.

Classification	PI in % (n)*	AI in % (n)*
B-cell		
Small B-cell MALT lymphoma	5.80 (10)	0.06 (10)
Large B-cell MALT lymphoma	52.81 (10)	0.24 (12)
Large B-cell lymphoma	58.85 (17)	1.36 (15)
Mantle cell lymphoma	30.97 (2)	1.93 (2)
T-cell		
Small T-cell lymphoma	18.13 (2)	1.25 (2)
Large B-cell lymphoma	43.40 (7)	1.93 (6)
Total	37.30 (49)	1.10 (47)

*(The numbers in parentheses are the number of evaluable cases for the different staining methods).

Results

Proliferation and apoptosis in B-cell lymphoma

The proliferation index as assessed by mib-1 immunostaining could be evaluated in 49 cases, apoptotic index using the TUNEL method in 47 cases. In the remaining cases adequate evaluation was not possible due to non-specific staining or loss of tissue by pretreatment methods. Table 8.1 lists the median values in the different entities, demonstrating that PI are higher in large cell lymphomas. Comparing the median PI of all large cell cases (MALT as well as non-MALT cases) with the small cell cases, large cell cases show a significantly higher proliferation (Figure

8.1a and 8.1b, see appendix) (Medians: 52.8 vs. 3.7%, $p=0.0025$, Mann-Whitney U test). No difference in PI between large cell NHL with and without a MALT component was observed. Apoptotic Indices are also higher in the large cell lymphomas (Figure 8.1c, see appendix). Comparison of all small with all large cell cases shows a significantly higher AI in the large cell cases (Medians: 1.08% vs. 0.05%, $p=0.019$, Mann-Whitney U test). Large cell MALT lymphomas demonstrate a lower median AI (0.24% vs. 1.36%) compared to large cell lymphomas without a MALT component (Mann-Whitney U-test, $p=0.14$).

Staining for the bcl-2 protein was evaluable in thirty-eight cases of which 14 cases (36.8%) did not express bcl-2, 13 cases (34.27%) had an intermediate expression and 11 cases (28.9%) a high expression. Small cell cases demonstrated mainly intermediate (55.6%) or high expression of bcl-2 (33.3%), only 1 case (11.1%) being negative for bcl-2. The majority of large cell cases was bcl-2 negative (44.8%), 27.6% demonstrated an intermediate and 27.6% a high expression of bcl-2. Staining for the P53-protein was evaluable in all cases, but only 8 cases showed more than 30% p53 positive cells. Seven of these cases were large cell lymphomas, (3 large cell MALT cases, 4 non-MALT cases). Although the number of p53 positive cases was higher in large cell cases than in small cell cases, this was not statistically significant (Fisher's Exact Test, $p=0.41$).

Analysing the relation between cell proliferation and apoptosis, we found a positive correlation, as shown in figure 8.2. As can be noticed from the figure, small cell cases show both low AI and PI whereas large cell cases are spreading out towards higher AI and PI. The Spearman correlationcoefficient 0.568 ($p<0.001$) represents all cases.

Figure 8.2 Correlation between proliferative index (PI) and apoptotic index (AI) in primary intestinal small (●) and large (○) B-cell non-Hodgkin's lymphoma.

We furthermore analyzed the relation between bcl-2 and p53 expression on the one hand and proliferative and apoptotic indices on the other hand. Figure 8.3 shows that the PI decreases with increasing bcl-2 expression (Kruskal-Wallis 1-Way Anova, $p=0.0004$). In figure 8.4, the relation between bcl-2 and AI is shown. Bcl-2 negative cases and bcl-2 intermediate cases had significantly higher AIs compared to cases with a high bcl-2 expression (Mann-Whitney U test: $p=0.0002$ and $p=0.0038$, respectively). Due to the low number of p53 positive cases we could not establish a relationship between p53 positivity and proliferative and apoptotic indices.

All large cell cases showed more than 10% diffusely intermingled blasts, whereas small cell lymphomas showed less than 10%. Figure 8.5 shows the relationship between the proliferation index and the percentage of blasts.

Figure 8.3 Relation between bcl-2 expression pattern (negative, intermediate or high) and proliferative index (PI) in primary intestinal B-cell NHL.

Figure 8.4 Association of bcl-2 expression pattern (negative, intermediate or high) and apoptotic index in small (●) and large (○) cell primary intestinal B-cell NHL.

Figure 8.5 Histologic grading classification (percentage of diffusely intermingled blasts) in relation to the proliferative index (PI) as determined by mib-1 immunohistochemistry.

Results in T-cell lymphomas & Mantle cell lymphomas

Median AI and PI in T-cell lymphomas were not significantly different from AI and PI in their B-cell counterparts. None of the T-cell lymphomas showed a high bcl-2 staining pattern, four showed an intermediate staining pattern and five were bcl-2 negative. P53 protein overexpression was detected in one T-cell lymphoma. No significant correlations between AI and PI; bcl-2 and AI; and bcl-2 and PI were found, mainly due to the low number of T-cell lymphomas ($n=9$). Mantle cell lymphomas showed a PI in between the PI in small and large cell lymphomas and an AI resembling large cell lymphomas. P53 overexpression was not detected.

Analysis of the different localisations of the intestinal tract

PI and AI were not significantly different in the diverse localisations in the digestive tract, the median values being respectively 50.00%/1.39% in small intestinal, 23.1%/1.10% in ileocecal, 23.60%/0.68% in colonic and 12.40%/0.60% in rectal lymphoma. The different localisations did not show different expression patterns for the bcl-2 protein.

Discussion

The present study was the first one to investigate the role of proliferation and apoptosis in primary intestinal lymphomas. We found that small cell intestinal lymphomas have a significantly lower PI when compared to large cell intestinal lymphomas. Furthermore, our data show that apoptotic indices in intestinal

lymphomas are higher in large cell lymphomas as well as in bcl-2 negative lymphoma and that there is a positive correlation between cell proliferation and apoptosis in these lymphomas.

Although a number of studies have investigated and interrelated proliferation and apoptosis in nodal non-Hodgkin's lymphomas^{26,27}, studies on extranodal, and in particular, intestinal lymphomas are scarce^{28,29}, mainly presenting data on proliferation. The data on proliferation in our study are in accordance with the data of Woods et al., who investigated proliferation in primary gastrointestinal lymphoma and found a strong correlation between PCNA (Proliferating Cell Nuclear Antigen) score and histological grade of the tumour²⁹. Rivas et al. used Ki-67 expression to assess proliferation in ten cases of MALT lymphoma, of which four were intestinal and also found that Ki-67 positivity was lower in low grade lymphoma compared to high grade lymphoma (range 5%-30% vs. 50%-70%)²⁸.

In recent years, it has become clear that in order to get an impression of tumour growth, the net balance between cell proliferation and cell death is important^{27,30,31}. Apoptosis is one of the major mechanisms of cell death, as described by Wyllie³². In nodal non-Hodgkin's lymphomas, studies on cell turnover have shown a highly significant difference between the cell turnover index (which was defined by the sum of the Apoptotic and the Mitotic Index) in low and high grade lymphomas³³ and furthermore, it has been found that in nodal non-Hodgkin's lymphoma, there is a positive correlation between the apoptotic and mitotic indices and both cell turnover characteristics correlated with the histological grade²⁶.

In primary gastrointestinal lymphoma the role of cell proliferation and apoptosis has been investigated by Du et al. who mainly studied gastric lymphomas and included five intestinal MALT lymphomas⁸ and in our own previous study on 72 primary gastric lymphomas⁹. Results of both studies are comparable to studies in primary nodal lymphomas in that low proliferative and apoptotic indices in low grade primary gastric lymphomas and high PI and AI in high grade cases were found. Furthermore, rather loose, but positive correlations were observed between AI and PI, like in nodal lymphoma.

The proliferative and apoptotic indices in intestinal lymphoma noticed in this study, appeared to be related to small or large cell histology. In order to make a more objective classification of small and large cell lymphomas we also applied a histologic staging system for gastric lymphoma recently described by the Jong et al.²³. Our data validate this new gastric lymphoma classification also in intestinal lymphoma and also show that the percentage of 10% diffusely intermingled blasts is a reliable cut-off value for the subdivision in small and large cell lymphoma.

One of the differences between primary gastric and primary intestinal lymphomas in our series is the relatively large proportion of large B-cell lymphomas without a detectable small cell MALT component in intestinal lymphomas. Although according to the literature most primary intestinal B-cell lymphomas are of MALT

type^{34,35}, we failed to detect MALT features in 18 large B-cell lymphomas. Comparing large cell MALT lymphomas to large cell lymphomas without a MALT component, we found about the same PIs, but the AI in the large cell MALT lymphomas was lower (although not significant).

Investigation of the relation between bcl-2 expression and apoptosis demonstrated that cases with high expression of bcl-2 showed significantly lower rates of apoptosis as compared to cases negative for bcl-2, in keeping with the data indicating that bcl-2 can inhibit apoptosis¹³. The presence of several bcl-2 negative and bcl-2 intermediate cases with very low apoptotic indices suggests that other factors besides bcl-2 also play a role in the regulation of apoptosis in intestinal lymphoma.

p53, which in its mutant form fails to induce apoptosis, does not show a relation with apoptosis in the present study, since due to a small number of cases a proper analysis could not be performed. In addition, it must be taken into account, that we were not really informed on the presence of mutations in the p53 gene³⁶. However, the Do-7 antibody has been shown to be relatively reliable for the detection of p53 gene mutations by immunohistochemistry³⁷ especially when combined with the right pretreatment method and using a cut-off point of more than 30% p53 positive cells^{34,25}.

Differences in proliferative and apoptotic indices between the different localisations in the gastrointestinal tract were not significant, small intestinal lymphoma showing the highest proliferation rate whereas the rectum demonstrated the lowest.

Analysis of the T-cell lymphomas was hampered by their low number (n=9). In accordance with results in B-cell lymphomas, they displayed higher PI and AI in large cell cases. Only one case was positive for the p53 protein, in contrast to findings of Murray et al. who found expression of the p53 protein in 22 of 23 T-cell lymphomas³⁸. However, their study consisted of enteropathy associated T-cell lymphomas (EATL), which may account for the discrepancy as we did not observe EATL in our series. None of the T-cell lymphomas expressed high levels of bcl-2, maybe accounting for the higher AI in small T-cell lymphomas compared to small B-cell lymphomas, the majority of which does show high expression of bcl-2.

In conclusion, small cell primary intestinal non-Hodgkin's lymphomas show both significantly lower PI and AI compared to large cell lymphomas, and these two components of cell turnover are positively correlated. These findings are comparable to both gastric lymphomas as well as to nodal lymphomas. Therefore, cell turnover probably is not an important phenomenon explaining the different behaviour of nodal versus extranodal lymphoma varieties.

References

1. Dragosics B, Bauer P, Radaszkiewicz T. Primary gastrointestinal non-Hodgkin's lymphomas: a retrospective clinicopathological study of 150 cases. *Cancer* 1985;55:1060-1073.
2. Weingrad DN, Decosse JJ, Sherlock P, Straus D, Lieberman PH, Filippa DA. Primary gastrointestinal lymphoma: A 30-year review. *Cancer* 1982;49:1258-1265.
3. Weiss NS, Yang CP. Incidence of histologic types of cancer of the small intestine. *J Natl Cancer I* 1987;78:653-656.
4. DiSario JA, Burt RW, Kendrick ML, McWhorter WP. Colorectal cancers of rare histologic types compared with adenocarcinomas. *Dis Colon Rectum* 1994;37:1277-1280.
5. Zigelboim J, Larson MV. Primary colonic lymphoma. Clinical presentation, histopathological features, and outcome with combination chemotherapy. *Journal of Clin Gastroenterol* 1994;18:291-297.
6. Levine AM. Acquired immunodeficiency syndrome-related lymphoma. *Blood* 1992;80:8-20.
7. Nalesnik MA, Jaffe R, Starzl TE. The pathology of posttransplant lymphoproliferative disorders occurring in the setting of cyclosporine A-prednisone immunosuppression. *Am J Pathol* 1988;133:173-192.
8. Du M, Singh N, Husseuin A, Isaacson PG, Pan L. Positive correlation between apoptotic and proliferative indices in gastro-intestinal lymphomas of mucosa-associated lymphoid tissue (MALT). *J Pathol* 1996;178:379-384.
9. Gisbertz IAM, Schouten HC, Bot FJ, Arends J-W. Proliferation and apoptosis in primary gastric B-cell non-Hodgkin's lymphoma. *Histopathology* 1997;30:152-159.
10. Gavrieli Y, Sherman Y, Ben-Sasson SA. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J Cell Biol* 1992;119:493-501.
11. Wijsman JH, Jonker RR, Keijzer R, van de Velde CJH, Cornelisse CJ, van Dierendonck JH. A new method to detect apoptosis in paraffin sections: in situ end-labeling of fragmented DNA. *J Histochem Cytochem* 1993;41:7-12.
12. Chao DT, Linette GP, Boise LH, White LS, Thompson CB, Korsmeyer SJ. Bcl-x_L and Bcl-2 repress a common pathway of cell death. *J Exp Med* 1995;182:821-828.
13. Hockenbery DM. The bcl-2 gene and apoptosis. *Semin Immunol* 1992;4:413-420.
14. Mazel S, Burtrum D, Petrie HT. Regulation of cell division cycle progression by bcl-2 expression: a potential mechanism for inhibition of programmed cell death. *J Exp Med* 1996;183:2219-2226.
15. Agarwal ML, Agarwal A, Taylor WR, Stark GR. p53 controls both the G2/M and the G1 cell cycle checkpoints and mediates reversible growth arrest in human fibroblasts. *Proc Natl Acad Sci USA* 1995;92:8493-8497.
16. Gannon JV, Greaves R, Iggo R, Lane DP. Activating mutations in p53 produce a common conformational effect. A monoclonal antibody specific for the mutant form. *EMBO J* 1990;9:1595-1602.

17. Newcomb EW. P53 gene mutations in lymphoid diseases and their possible relevance to drug resistance. *Leukemia Lymphoma* 1995;17:211-221.
18. Magrath I. The pathogenesis of Burkitt's lymphoma. *Adv Cancer Res* 1990;55:133-170.
19. Rohatiner A, D'Amore F, Coiffier B, Crowther D, Gospodarowicz M, Isaacson P, Lister TA, Norton A, Salem P, Shipp M, Somer R. Report on a workshop convened to discuss the pathological and staging classifications of gastrointestinal tract lymphoma. *Ann Oncol* 1994;5:397-400.
20. Isaacson P, Wright DH. Malignant lymphoma of mucosa-associated lymphoid tissue. *Cancer* 1983;52:1410-1416.
21. Isaacson PG. Lymphomas of mucosa-associated lymphoid tissue (MALT). *Histopathology* 1990;16:617-619.
22. Isaacson PG, Spencer J. Malignant lymphoma of mucosa-associated lymphoid tissue. *Histopathology* 1987;11:445-462.
23. DeJong D, Boot H, VanHeerde P, Hart GAM, Taal BG. Histological grading in gastric lymphoma: pretreatment criteria and clinical relevance. *Gastroenterology* 1997;112:1466-1474.
24. Baas IO, Mulder JWR, Offerhaus GJA, Vogelstein B, Hamilton SR. An evaluation of six antibodies for immunohistochemistry of mutant p53 gene product in archival colorectal neoplasms. *J Pathol* 1994;172:5-12.
25. Hall PA, Lane DP. p53 in tumour pathology: can we trust immunohistochemistry? - revisited! *J Pathol* 1994;172:1-4.
26. Leoncini L, DelVecchio MT, Megha T, Barbini D, Galieni P, Pileri S, Sabattini E, Gherlinzoni F, Tosi P, Kraft R, Cottier H. Correlations between apoptotic and proliferative indices in malignant non-Hodgkin's lymphomas. *Am J Pathol* 1993;142:755-763.
27. Marx J. Cell death studies yield cancer clues. *Science* 1993;259:760-761.
28. Rivas C, Echezarreta G, Garcia R, Santos M, Santon A, Robledo M, Benitez J, Oliva H, Delsol G. A multiparametric study of malignant lymphoma of mucosa associated lymphoid tissue. *Leukemia Lymphoma* 1992;8:87-96.
29. Woods AL, Hall PA, Shepherd NA, Hanby AM, Waseem NH, Lane DP, Levison DA. The assesment of proliferating nuclear cell antigen (PCNA) immunostaining in primary gastrointestinal lymphomas and its relationship to histological grade, S + G2 + M phase fraction (flow cytometric analysis) and prognosis. *Histopathology* 1991;19:21-27.
30. Kerr JFR, Winterford CM, Harmon BV. Apoptosis. Its significance in cancer and cancer therapy. *Cancer* 1994;73:2013-2026.
31. Sedivy R. The potential role of apoptosis (programmed cell death) in a chaotic determined carcinogenesis. *Med Hypotheses* 1996;46:455-457.
32. Wyllie AH, Kerr JRR, Currie AR. Cell death: the significance of apoptosis. *Int Rev Cytol* 1980;68:251.
33. Spina D, Leoncini L, DelVecchio MT, Megha T, Minacci C, Poggi SA, Pileri SA, Tosi P, Kraft R, Laissue JA, Cottier H. Low versus high cell turnover in diffusely growing non-Hodgkin's lymphomas. *J Pathol* 1995;177:335-341.

34. Kojima M, Nakamura S, Kurabayashi Y, Shimizu K, Mosomura Y, Ohno Y, Itoh H, Yoshida K, Sano T, Sakurai S, Iwasaka S, Fujisaki M, Suchi T, Joshita T. Primary malignant lymphoma of the intestine: Clinicopathologic and immunohistochemical studies of 39 cases. *Pathol Int* 1995;45:123-30.
35. Radaszewicz T, Dragosics B, Bauer P. Gastrointestinal malignant lymphomas of the mucosa-associated lymphoid tissue: Factors relevant to prognosis. *Gastroenterology* 1992; 102: 1628-38.
36. Martinez-Delgado B, Robledo M, Arranz E, Infantes F, Echezarreta G, Marcos B, Sanz C, Rivas C, Benitez J. Correlation between mutations in p53 gene and protein expression in human lymphomas. *Am J Hematol* 1997;55:1-8.
37. Bonsing BA, Corver WE, Gorsira MCB, van Vliet M, Oud PS, Cornelisse CJ, Fleuren GJ. Specificity of seven monoclonal antibodies against p53 evaluated with Western Blotting, immunohistochemistry, confocal laser scanning, microscopy and flow cytometry. *Cytometry* 1997;28:11-24.
38. Murray A, Cuevas EC, Jones DB, Wright DH. Study of the immunohistochemistry and T cell clonality of enteropathy-associated T cell lymphoma. *Am J Pathol* 1995;146:509-519.

Chapter 9

Primary intestinal non-Hodgkin's lymphoma: clinical description of 39 consecutive cases and correlations with cell turnover parameters

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Abstract

Background

Primary intestinal non-Hodgkin's lymphoma (NHL) is an uncommon disease, with heterogeneous localisations and histologic appearances. Retrospectively, a population based series was studied to describe the clinical course and to investigate the clinical consequences of histological classification.

Patients and Methods

Clinical data (presentation, staging, therapy, recurrence, survival), histology, proliferative and apoptotic indices, expression of p53 protein of thirty-nine patients with intestinal NHL were collected.

Results

Abdominal pain, weight loss, diarrhoea and intestinal bleeding were the most frequently observed presenting signs. The majority of the patients presented in stage I (16) and II (13), two patients presented in stage III and six in stage IV. No significant differences for small and large cell lymphomas were observed. Twenty-five patients died, with seventeen of lymphoma (median survival in the latter group: 68 days, mean 389 days). None of the patients with small cell NHL died of lymphoma, thus showing a significantly better survival than large cell cases. Expression of the p53 protein also was a significant indicator of poor survival.

Conclusions

Large B-cell primary intestinal NHL had a poorer survival compared to small B-cell non-Hodgkin's lymphoma. Furthermore, expression of the p53 protein was associated with poorer survival.

Introduction

Intestinal non-Hodgkin's lymphomas (NHL) account for one-third of primary gastrointestinal NHL¹. In the past, primary gastric and primary intestinal NHL were generally studied as one group²⁻⁴. Nowadays, primary intestinal lymphoma is often studied separately from gastric NHL, because of differences in presentation, therapy and a generally worse prognosis⁵.

Due to their very rare occurrence and, furthermore, their heterogeneity, study of primary intestinal lymphomas is difficult. Intestinal NHL originate in different parts of the intestinal tract, both B- and T-cell lymphomas occur and different histopathological subtypes are found. Most studies focus on one of these aspects or on one treatment modality. To avoid bias due to selection of patients we studied a retrospective series from a population based registry in an area of about 1,000,000 inhabitants over a time span of 9 years. In a previous study we analyzed the role of proliferation and apoptosis in primary intestinal lymphomas⁶. Generally, proliferative and apoptotic rates were higher in large cell lymphoma compared to small cell lymphoma. Proliferation has been shown to be of prognostic value in nodal non-Hodgkin's lymphoma and other tumours which prompted us to analyze this relation also in intestinal NHL. Since tumour growth is regulated by both proliferation as well as apoptosis, we also investigated the role of apoptosis and two gene products involved in the regulation of apoptosis. Here we describe the clinical presentations, treatment and prognosis of thirty-nine patients treated in different hospitals during the period of 1986 till 1994. Moreover, we tried to determine whether the histological subtype of intestinal lymphoma and certain clinical parameters related to the ultimate prognosis in this patient group.

Patients and Methods

Patients

The Limburg Working Party on lymphoma records all new lymphomas diagnosed in the province of Limburg. Histology is studied by all participating pathologists prior to a consensus meeting in which the cases are discussed and a diagnosis is made. All intestinal lymphomas diagnosed between July 1986 and December 1994 were selected from this registry (n=50). Further screening of pathology reports, revision of the material and clinical data led to the exclusion of cases with secondary involvement of the intestinal tract by nodal non-Hodgkin's lymphoma (n=5) and cases without an evident association with the mucosa (mesenterial NHL, n=2). Burkitt's lymphomas (n=4) were also excluded because of their specific pathogenesis. Disseminated cases were included if the predominant lesion was

located in the intestines. This resulted in a final number of 39 cases for further investigation. Haematoxylin and eosin stained slides of these cases were reviewed by two pathologists (F.J.B and J.W.A.) and classified as small or large B-cell lymphoma with or without MALT features, small or large T-cell lymphoma and mantle cell lymphoma, according to the classification proposed at the fifth international conference of lymphoma in Lugano in 1993⁷.

Clinical information on presenting symptoms and signs, history, staging, therapy, and outcome was gathered from clinical and outpatient files. Clinical staging was performed according to the Ann Arbor classification modified by Musshoff⁸. Patients were treated according to the preference of the local physician, depending on the existing protocols in each hospital at the time of diagnosis.

Methods

Data on proliferative and apoptotic indices, bcl-2 and p53 protein expression studied earlier were also correlated to outcome of the patients. Immunohistochemical reactions and TUNEL were performed as described before⁹. Briefly, the proliferative index (PI) was determined by the relative frequency of mib-1 immunoreactive cells, apoptotic indices (AI) by the relative frequency of terminal deoxynucleotidyl-transferase (TdT)-mediated nick end labelling (TUNEL)-positive nuclei, both performed on 2 micron tissue sections. The expression of the p53 protein was investigated with the Do-7 antibody and was scored positive when more than 30% of the nuclei showed reactivity, bcl-2 expression patterns using clone 124 were classified as negative, intermediate and high expression.

Statistics

Patients were followed from diagnosis up till July 1996 or to the date of the last follow-up evaluation or death. The median follow-up was 384 days (mean 896.36), ranging from 1-3579 days. The Mann-Whitney U test was used to compare values of continuous variables between small and large B-cell lymphomas. The association between categorical variables for small and large B-cell NHL was evaluated with a chi-square (χ^2) test or with Fisher's exact test when the samples were too small. The Overall Survival (OS) was calculated from the date of diagnosis until the date of last follow-up or death. Patients who died during treatment or due to consequences of treatment were considered as deaths due to lymphoma. Patients who died because of non-lymphoma related causes were censored. Furthermore, because the number of patients dying from treatment-related causes was rather high, we also calculated survival curves in which both non-lymphoma related as well as treatment-related causes of death were censored. Survival curves were calculated according to the Kaplan-Meier method and differences between curves were evaluated using the log-rank test. Survival curves were compared between patients

with small and large cell histology, stage I and II-III-IV disease, high and low proliferative tumours, high and low apoptotic tumours, and, p53 positive and negative tumours. These factors were compared in both the B-cell lymphomas only as well as in the total series (survival curves for each factor are separated for phenotype). Survival of B- and T-cell lymphomas was also compared.

Results

Clinical presentation

Table 9.1 shows some general patient characteristics. The median age of the patients at diagnosis was 60 years (range 35-88). Twenty-five patients were male, 14 patients were female. The majority of lymphomas were found in the small intestines ($n=17$, 43.6%), followed by the colon and ileocecal region (both 7 cases, 17.9%). Five lymphomas were localized in the rectum (12.8%), two in the appendix (5.1%) and in one case there was involvement throughout the intestinal tract at presentation (2.6%). The most frequent symptoms and signs at presentation were abdominal pain, weight loss, diarrhoea, nausea and intestinal bleeding. Physical examination showed splenomegaly in 4 and hepatomegaly in 2 patients. Peptic ulcer disease was the most often reported relevant co-existing disease in the past medical history (in 5 cases). No significant differences between small and large B-cell lymphomas were observed for the variables described above.

Histological classification

Eight cases were classified as small B-cell MALT lymphoma, 10 cases as large B-cell MALT lymphoma, 13 cases as large B-cell lymphoma without a small cell MALT component, 6 cases as T-cell lymphoma (4 large cell, 2 small cell) and 2 cases as mantle cell lymphomas.

Clinical staging

Staging procedures included chest-X-ray, CT scan of the abdomen (in 29 patients, 74.4%), bone marrow aspirate (in 24 patients, 61.5%) and ear nose and throat examination (in 15 patients, 38.5%). The majority of patients presented in stages I ($n=16$) or II ($n=13$). Eight patients presented with disseminated disease (2 with stage III and 6 with stage IV). The latter showed bonemarrow infiltration (3 patients), involvement of the liver (2 patients) or both (1 patient). In two patients there was no information on staging procedures. After surgery, positive lymph nodes were found in 4 clinically stage I patients, which were subsequently upstaged to pathological stage II.

Table 9.1 Primary intestinal lymphoma - age, sex, localisation, presentation, stage, history.

	B-cell intestinal NHL ¹ Small cell	Large cell	T-cell NHL and mantle cell NHL	Total
Number of patients	8	23	8	39
Age mean (range)	56 (36-75)	64 (35-88)	62(43-87)	62 (35-88)
Sex M/F	4/4	14/9	7/1	25/14
Localisation (n,%)				
small intestine	3 (37.5%)	9 (39.1%)	5 (62.5%)	17 (43.6%)
ileocecal	2 (25.0%)	5 (21.7%)	0	7 (17.9%)
appendix	0	2 (8.7%)	0	2 (5.1%)
colon	2 (25.0%)	3 (13.0%)	2 (25.0%)	7 (17.9%)
rectum	1 (12.5%)	3 (13.0%)	1 (12.5%)	5 (12.8%)
miscellaneous	0	1 (4.3%)	0	1 (2.6%)
Presentation (n,%)				
Abdominal pain	3 (37.5%)	16 (69.6%)	4 (57.1%)	23 (60.5%)
Weight loss	2 (25.0%)	12 (52.2%)	4 (57.1%)	17 (44.7%)
Diarrhoea	4 (50.0%)	8 (34.8%)	4 (57.1%)	16 (42.1%)
Nausea	2 (25.0%)	8 (34.8%)	1 (14.3%)	11 (28.9%)
GI blood loss	3 (37.5%)	5 (22.7%) ²	3 (42.9%)	11 (28.9%)
Dyspepsia	4 (50.0%)	4 (17.4%)	1 (14.3%)	9 (23.7%)
Perforation	0	6 (26.1%)	3 (42.9%)	9 (23.7%)
Obstruction	3 (37.5%)	2 (8.7%)	3 (42.9%)	8 (21.1%)
Anorexia	1 (12.5%)	6 (26.1%)	0	7 (18.4%)
Vomiting	1 (12.5%)	4 (17.4%)	0	5 (13.2%)
Fever	1 (12.5%)	2 (8.7%)	1 (14.3%)	4 (10.5%)
Transpiration	1 (12.5%)	3 (13.0%)	0	4 (10.5%)
No data	0	0	1	1
Physical examination				
Splenomegaly	0	3 (14.3%)	1 (14.3%)	4 (11.1%)
Hepatomegaly	0	1 (4.8%)	1 (14.3%)	2 (5.6%)
No data	0	2	1	3
Stage				
I	4 (50.0%)	9 (39.1%)	3 (37.5%)	16 (41.0%)
II1	1 (12.5%)	1 (4.3%)	0	2 (5.1%)
II1e	1 (12.5%)	6 (26.1%)	1 (12.8%)	8 (20.5%)
II2	0	1 (4.3%)	0	1 (2.5%)
II2e	1 (12.5%)	1 (4.3%)	0	2 (5.1%)
IIIe	0	1 (4.3%)	0	1 (2.5%)
IIIs	0	1 (4.3%)	0	1 (2.5%)
IV	1 (12.5%)	3 (13.0%)	2 (25.0%)	6 (15.4%)
unknown	0	0	2 (25.0%)	2 (5.1%)
pathol. stage II	2 (25.0%)	2 (8.6%)	0	0
History				
Peptic ulcer dis.	1 (12.5%)	2 (8.7%)	2 (25.0%)	5 (12.8%)
Coeliac disease	0	1 (4.3%)	1 (12.5%)	2 (5.3%)
Villous atrophy	0	0	1 (12.5%)	1 (2.7%)

¹ Comparison of the variables in small and large cell histology showed no significant differences.

² The presence of GI blood loss was only known in 22 patients.

Therapy

The following therapy was given: 2 patients received no therapy (refused), 29 patients were treated by surgery of which 15 patients had no adjuvant chemo- or radiotherapy; 13 patients received additional chemotherapy and 1 patient was treated with all three modalities. In the remaining 8 cases 7 patients had chemotherapy only and one patient received radiotherapy only. Of the 29 patients treated with surgery 9 showed complications (31.0%), being infections (5), perforation (1), ileus (1), pulmonary embolus (1) and 1 patient had to be mechanically ventilated after surgery. Eventually 7 patients died of surgery-related causes. Thirteen of the twenty patients undergoing chemotherapy received an adriamycin-containing regimen, the mean number of regimens given was six. Complications in chemotherapy were seen in 9 patients (45.0%), cytopenia was observed in 7 patients, with sepsis 1 patient; 1 patient developed a toxic neuropathy and 1 patient venous thrombosis. No perforations were observed. Two patients died of their complications (one of sepsis and one as a result of chemotherapy induced cytopenia without further information on the cause of death). The patients treated with radiotherapy had no complications.

Outcome of the patients

In 13 of the 29 patients receiving surgical therapy positive lymph nodes were found and in 7 cases the resection margins were not free of lymphoma. In all but one case, information on the status of the tumour after therapy was found in the clinical files.

Figure 9.1 Survival in small (▲) and large (●) B-cell and small (○) and large (■) T-cell primary intestinal non-Hodgkin's lymphoma. The difference between small and large cell NHL in general (both B- and T-cell lymphomas) was significant (Log rank test, $p=0.015$). The difference in survival in between small and large B-cell NHL only was also significant (Log rank test, $p=0.0127$).

Table 9.2 Presentation of individual cases.

no	age/ sex	localisation	stage	therapy	recurrent disease	survival (days)/status	Cause of death
Small B-cell MALT lymphoma							
1	55/♂	small intestine	II1	C	-	382	dead without disease (DWD)
2	73/♀	small intestine	IIIe	S/C	ileum	1743 +	
3	75/♂	small intestine	I	S	-	1242 +	
4	36/♀	ileocecal	I(> IIp)	S	-	3579 +	
5	50/♀	ileocecal	I(> IIp)	S	-	1308 +	
6	52/♂	colon	IV	C	-	1071 +	
7	70/♀	colon	II2e	C	-	1852	DWD (myocardial infarction)
8	41/♂	rectum	I	R	-	790	AIDS (DWD)
Large B-cell lymphoma with a MALT component							
9	53/♂	small intestine	IIIe	S/C	-	3023 +	
10	56/♂	small intestine	I	S/C	distant	307	myocardial infarction (DWD)
11	65/♀	small intestine	I	S	-	3	complicated surgery (DOD)
12	67/♀	small intestine	I	S	cauda eq.	200 +	
13	77/♂	small intestine	I	S	-	1	pulmonary embolus (DOD)
14	35/♀	ileocecal	IIIe	S/C	local	1434	progressive disease (DOD)
15	64/♂	ileocecal	IIIe	S/C	-	3101 +	
16	56/♂	colon	IIIe	S/C	-	209	progressive disease (DOD)
17	60/♀	colon	II2	C	-	87	progressive disease (DOD)
18	88/♂	rectum	IV	C	-	11	progressive disease (DOD)
Large B-cell lymphoma							
19	44/♂	small intestine	IIIe	S/C/R	skin-kidney/bone	866	progressive disease (DOD)
20	57/♂	small intestine	I(> IIp)	S/C	-	196 +	
21	79/♀	small intestine	I(> IIp)	S	stomach	931	dead of recurrent disease (DOD)
22	84/♀	small intestine	IIIe	S/C	-	68	progressive disease (DOD)
23	46/♂	ileocecal	I	S	-	2070 +	
24	54/♀	ileocecal	IIIe	S/C	local	668	complication chemotherapy (DOD)
25	83/♂	ileocecal	II2e	S	yes	2186	progressive disease, cachexia (DOD)
26	65/♀	appendix	III	S	-	384	respiratory failure (DWD)
27	74/♂	appendix	I	S/C	-	1190 +	
28	73/♂	colon	IV	C	-	217 +	
29	36/♂	rectum	IV	C	orbit, testis, skin	160	dead of disease, AIDS (DWD)
30	85/♀	rectum	I	no th.	-	94	myocardial infarction (DWD)
31	60/♂	miscellaneous	III	S/C	-	75	sepsis, perforation (DOD)
Small T-cell lymphoma							
32	67/♂	small intestine	I	S	-	2223	cardiomyopathy (DWD)
33	87/♂	small intestine	I	S	-	6	complicated surgery (DOD)
Large T-cell lymphoma							
34	43/♀	small intestine	IV	S/C	-	34	sepsis after chemotherapy (DOD)
35	52/♂	small intestine		S	-	11	pneumonia post-surgery (DOD)
36	70/♂	colon	IV	S/C	-	736 +	
37	54/♂	colon	I	S	-	8	complicated surgery (DOD)
Mantle cell lymphoma							
38	46/♂	small intestine	IIIe	S	-	16	complicated surgery (DOD)
39	75/♂	rectum		no ?	-	2476 +	

(>IIp) = restaging after surgical detection of positive lymph nodes; S = surgery; C = chemotherapy; R = radiotherapy; + = patients alive at last follow-up; DOD = dead of disease; DWD = dead without disease.

According to this information, 20 patients had a complete remission, of which nine showed clinical relapse (8 large cell and 1 small cell patient), in one the site of recurrence was unknown, the other sites are shown in table 9.2. Eighteen patients showed no remission. Small cell lymphomas demonstrated the highest complete remission rate, being 75%, large T-cell lymphoma the lowest (25%).

Twenty-five of the 39 patients died, 8 of which of unrelated causes (cardiac disease in 4, AIDS in 2, respiratory failure in 1, 1 unknown). Seventeen patients died of disease-related causes, nine of which were treatment-related (Median survival 68 days, mean 389 days, range 1-2186). Fourteen patients were alive at the last follow-up, their median follow-up time was 1275 days (mean 1582), range 196-3579. None of the patients with small B-cell lymphomas died of a lymphoma-related cause and in this way had a significant better survival than large cell cases (Figure 9.1). Censoring of the treatment related deaths led to comparable results. Stage at presentation was no discriminating factor, (logrank test in B+T-cell lymphoma, $p=0.4334$; in B-cell lymphoma: $p=0.3293$). The difference in prognosis between patients presenting in stage I ($n=16$) vs those in stage II, III and IV ($n=21$) increased when treatment related deaths were excluded (in B-cell lymphoma: $p=0.1452$). In figure 9.2, the survival curves for B- and T-cell lymphomas are shown. Although, initially, T-cell lymphomas have a worse prognosis, there was no statistically significant difference ($p=0.0825$). All deaths due to T-cell lymphoma were due to treatment.

Figure 9.2 Survival in primary intestinal lymphoma according to B (●) or T(○)-cell phenotype. (Log rank test, $p=0.0825$).

Correlations between survival and cell turnover

In a previous study we concluded that proliferative and apoptotic indices are significantly higher in large cell intestinal lymphomas compared to small cell lymphomas. Also in this series we detected this difference (Table 9.3). In the present study we related these data to clinical outcome in B-cell lymphomas. Proliferative indices did not discriminate between groups with good and poor survival (Logrank test: B&T-cell NHL:p=0.2495; B-cell NHL:p=0.1402). Figure 9.3 shows the curves for high and low apoptotic indices in both B- and T-cell NHL. Apoptotic indices showed a significant survival difference in the total series (p=0.0029), but not in B-cell NHL only (p=0.1344). When the treatment related deaths were censored from the total (B&T-cell NHL) series, the AI was no discriminant factor anymore. P53 positive cases had a poorer prognosis than p53 negative cases, both in the B-cell group (p=0.0013), as well as in the whole series (p=0.0388). Figure 9.4 shows the curves for p53 positive and negative B-cell NHL and p53 negative T-cell NHL (none of the T-cell NHL showed p53 protein expression). When treatment-related deaths were censored, this difference gained in significance (p=0.0001).

Table 9.3 Proliferative Index (PI) and Apoptotic Index (AI) in primary intestinal small and large cell lymphoma.

	Small cell	Large cell	p
Proliferation Index	13.56	59.23	0.005
Apoptotic Index	0.24	1.57	0.012

Figure 9.3 Survival in primary B- and T-cell lymphomas with high and low apoptotic indices.(▲ B-cell < median AI; ● B-cell > median AI; ○ T-cell < median AI; ■ T-cell > median AI). Patients with lymphomas displaying AIs above the median value had a significantly worse prognosis (Log rank test, total series, p=0.0029).

Figure 9.4 Survival in p53-positive (●) and negative (▲) B-cell NHL and in p53-negative T-cell lymphomas (○). P53 positive NHL showed a significant worse prognosis than p53 negative NHL (Log rank test, total series, $p = 0.038$).

Discussion

This study intended to describe the clinical features of consecutive cases of primary intestinal lymphoma, in a population-based study. The distribution of lymphoma throughout the intestinal tract reflects that described in the literature: the small intestines were the most often affected, followed by the colon, ileocecum and rectum.

Primary intestinal non-Hodgkin's lymphoma has often been studied in combination with gastric lymphoma^{3,4,10,11}. Also, they are often subdivided either by histology: mucosal mantle cell lymphoma (or lymphomatous polyposis)^{12,13}, enteropathy-associated T-cell lymphoma¹⁴⁻¹⁶, and immunoproliferative small intestinal disease^{17,18}; or by localisation: small intestine^{19,21} and colon- and rectum-lymphomas²²⁻²⁶.

Our finding that 35% of the B-cell lymphomas were small cell lymphomas of MALT is in concordance with other findings²⁷. Twenty percent of the cases were T-cell lymphomas, however, only one of these patients suffered from coeliac disease before diagnosis and features of EATL could not be recognized histologically. Interpreting these relative percentages, it should be borne in mind that Burkitt's lymphoma was excluded, diminishing the number of large B-cell lymphomas.

Over 60% of the patients presented with pain, whereas weight loss and diarrhoea were also frequently reported. Weight loss is probably a reflection of local problems and therefore must be cautiously interpreted as a B-symptom. Other B-symptoms (fever/drenching sweats) were infrequently noticed. Nine patients presented with perforation, three of which were T-cell lymphomas.

Physical examination, like in other studies, revealed no important aberrations. The

majority of patients presented in stage I. No association between survival and stage at presentation was found, in contrast to other studies.

The majority of the patients were treated surgically, and about half of these patients also received chemotherapy. A further eight patients received chemotherapy without surgery. It is noteworthy that none of the chemotherapy treated patients developed perforations, which is a feared complication of chemotherapy in gastrointestinal lymphoma.

Histology has often been shown to be important in the prediction of patient outcome. Indeed, also in this study, patients with small cell lymphomas had a significantly better prognosis than patients with large cell lymphomas. In fact, none of the patients with small cell lymphoma observed in this study died of lymphoma-related causes.

Analysis of the correlations between cell turnover parameters and survival in this unselected series was another aim of this study. The prognostic implications of a high proliferative index have been studied in human cancers, including non-Hodgkin's lymphoma^{28,29}. In recent years, apoptosis has been recognized as another important cell turnover parameter³⁰. In nodal lymphomas these features have been studied in relation to survival and it has been found that high apoptotic indices were correlated with a worse survival³¹. We studied these parameters in intestinal lymphomas and could not demonstrate a relationship of proliferation and survival in these lymphomas. This absence of correlation between proliferation indices and prognosis may be explained by the relatively high treatment related death rates. Higher apoptotic indices, however, were associated with a poor prognosis, like in nodal lymphoma. This apparently paradoxical finding - increased cell death should be associated with decrease in tumour size - is probably explained by the fact that high apoptotic rates were correlated with large cell histology and T-cell phenotype. The latter explains the loss of significance when treatment related deaths were excluded (all patients with T-cell NHL who died of lymphoma, died of treatment related causes).

P53 mutations are correlated with prognosis in many human tumours^{32,33}. The protein generated by the mutated p53 gene is accumulated in the cell, and can be studied by immunohistochemistry in this way³⁴. However, it has been shown that the p53 protein expression in lymphoma does not always correlate with mutations in the gene^{35,36}. Also in this series of primary intestinal lymphomas, the presence of accumulated p53 protein in more than 30% of the lymphoma cells was associated with poor prognosis, although the number of p53 protein positive lymphomas was very low.

In conclusion, large cell histology, and expression of the mutant p53 protein are, like in nodal lymphoma, worse prognostic factors in primary intestinal lymphoma. High apoptotic indices are also correlated with poor prognosis, but this is merely because high apoptotic indices are correlated with large cell histology and T-cell phenotype.

References

1. Turowski GA, Basson MD. Primary malignant lymphoma of the intestine. *Am J Surg* 1995;169:433-441.
2. Isaacson PG. Gastrointestinal lymphoma. *Hum Pathol* 1994;25:1020-1029.
3. Lewin KJ, Ranchod M, Dorfman RF. Lymphomas of the gastrointestinal tract. A study of 117 cases presenting with gastrointestinal disease. *Cancer* 1978;42:693-707.
4. Hall PA, Levison DA. Malignant lymphoma in the gastrointestinal tract. *Sem Diagn Pathol* 1991;8:163-177.
5. Rossi A, Rotahiner AZS, Lister TA. Primary gastro-intestinal non-Hodgkin's lymphoma: Still an unresolved question ? *Ann Oncol* 1993;4:802-803.
6. Gisbertz IAM, Schouten HC, Bot FJ, Arends JW. Cell turnover parameters in primary intestinal non-Hodgkin's lymphoma related to small and large cell varieties. *Cancer in press*
7. Rohatiner A, D'Amore F, Coiffier B, Crowther D, Gospodarowicz M, Isaacson P, Lister TA, Norton A, Salem P, Shipp M, Somer R. Report on a workshop convened to discuss the pathological and staging classifications of gastrointestinal tract lymphoma. *Ann Oncol* 1994;5:397-400.
8. Musshoff K. Klinische Stadieneinteilung der Nicht-Hodgkin-Lymphome. *Strahlentherapie* 1977;153:218-222.
9. Gisbertz IAM, Schouten HC, Bot FJ, Arends J-W. Proliferation and apoptosis in primary gastric B-cell non-Hodgkin's lymphoma. *Histopathology* 1997;30:152-159.
10. Van Krieken JHJM, Otter R, Hermans J, van Groningen K, Spaander PJ, van de Sandt MM, Keuning JF, Kluin PhM. Malignant lymphoma of the gastrointestinal tract and mesentery. *Am J Pathol* 1989;135:281-289.
11. Otter R, Bieger P, Hermans J, Willemze R. Primary gastrointestinal non-Hodgkin's lymphoma in a population-based registry. *Br J Cancer* 1989;60:745-50.
12. Ruskoné-Fourmestreaux A, Delmer A, Lavergne A, Molina T, Brousse N, Andouin J, Rambaud JC. Multiple lymphomatous polyposis of the gastrointestinal tract: prospective clinicopathological study of 31 cases. *Gastroenterology* 1997;112:7-16.
13. Fraga M, Lloret E, Sanchez-Verde L, Orradre JL, Campo E, Bosch F, Piris MA. Mucosal mantle cell (centrocytic) lymphomas. *Histopathology* 1995;26:413-422.
14. Pan L, Diss TC, Peng H, Lu Q, Wotherspoon AC, Thomas JA, Isaacson PG. Epstein-Barr virus (EBV) in enteropathy-associated T-cell lymphoma (EATL). *J Pathol* 1993;170:137-143.
15. Ilyas M, Niedobitek G, Aganhanggelou A, Barry RE, Read AE, Tierney R, Young LS, Rooney N. Non-Hodgkin's lymphoma, coeliac disease, and epstein-barr virus: a study of 13 cases of enteropathy-associated T- and B-cell lymphoma. *J Pathol* 1995;177:115-122.
16. Murray A, Cuevas EC, Jones DB, Wright DH. Study of the immunohistochemistry and T cell clonality of Enteropathy-associated T cell lymphoma. *Am J Pathol* 1995;146:509-519.
17. Price SK. Immunoproliferative small intestinal disease: A study of 13 cases with alpha heavy-chain disease. *Histopathology* 1990;17:7-17.

18. Khojasteh AK, Haghighi P. Immunoproliferative small intestinal disease: portrait of a potentially preventable cancer from the Third World. *Am J Med* 1990;89:483-490.
19. Morgan DR, Holgate CS, Dixon MF, Bird CC. Primary small intestinal lymphoma: a study of 39 cases. *J Pathol* 1985;147:211-221.
20. Jaser N, Sivula A. Primary small intestinal non-Hodgkin's lymphoma in Finland 1972-1977. *Ann Chir Gynaecol* 1991;80:250-257.
21. Rubesin SE, Gilchrist AM, Bronner M, Saul SH, Herlinger H, Grumbach K, Levine MS, Laufer I. Non-Hodgkin lymphoma of the small intestine. *RadioGraphics* 1990;10:985-998.
22. Kayabali I, Gökcora IH, Bacaci K, Cavusoglu T, Kutlu K. Primary colonic non-Hodgkin's lymphomas: A retrospective clinicopathological study of 14 cases. *Int Surg* 1992;77:195-197.
23. Zigelboim J, Larson MV. Primary colonic lymphoma. Clinical presentation, histopathological features, and outcome with combination chemotherapy. *J Clin Gastroenterol* 1994;18:291-297.
24. Aozasa K, Ohsawa M, Soma T, Sugiyama H, Yano Y, Shimano T, Hara K, Yamashita I, Masao I, Kuze S. Malignant lymphoma of the rectum. *Jpn J Clin Oncol* 1990;20:380-386.
25. Busch E, Rodriguez-Bigas M, Mamounas E, Barcos M, Petrelli NJ. Primary colorectal non-Hodgkin's lymphoma. *Ann Surg Oncol* 1994;1:222-228.
26. Jaser N. Primary ileocaecal and colorectal non-Hodgkin's lymphoma in Finland 1972-1977. *Ann Chir Gynaecol* 1991;80:329-335.
27. Kojima M, Nakamura S, Kurabayashi Y, Shimizu K, Mosomura Y, Ohno Y, Itoh H, Yoshida K, Sano T, Sakurai S, Iwasaka S, Fujisaki M, Suchi T, Joshita T.. Primary malignant lymphoma of the intestine: Clinicopathologic and immunohistochemical studies of 39 cases. *Pathol Int* 1995;45:123-130.
28. Czader M, Mazur J, Pettersson M, Liliemark J, Stromberg M, Christensson B, Tribukait B, Auer G, Ost A, Porwit A. Prognostic significance of proliferative and apoptotic fractions in low grade follicle center cell-derived non-Hodgkin's lymphomas. *Cancer* 1996;77:1180-1188.
29. Grogan TM, Lippman SM, Spier CM, Slymen DJ, Rybski JA, Rangel CS. Independent prognostic significance of a nuclear proliferation antigen in diffuse large cell lymphomas as determined by the monoclonal antibody Ki-67. *Blood* 1988;71:1157-1160.
30. Kerr JFR, Winterford CM, Harmon BV. Apoptosis. Its significance in cancer and cancer therapy. *Cancer* 1994;73:2013-2026.
31. Leoncini L, DelVecchio MT, Megha T, Barbini P, Galieni P, Pileri S, Sabattini E, Gherlinzoni F, Tosi P, Kraft R, Cottier H. Correlations between apoptotic and proliferative indices in malignant non-Hodgkin's lymphomas. *Am J Pathol* 1993;142:755-763.
32. Harris CC, Hollstein M. Clinical implications of the p53 tumor-suppressor gene. *New Engl J Med* 1993;329:1318-1327.
33. Fung CY, Fisher DE. p53: From molecular mechanisms to prognosis in cancer. *J Clin Oncol* 1995;13:808-811.

34. Bonsing BA, Corver WE, Gorsira MCB, van Vliet M, Oud PS, Cornelisse CJ, Fleuren GJ. Specificity of seven monoclonal antibodies against p53 evaluated with Western Blotting, immunohistochemistry, confocal laser scanning, microscopy and flow cytometry. *Cytometry* 1997;28:11-24.
35. Martinez-Delgado B, Robledo M, Arranz E, Infantes F, Echezarreta G, Marcos B, Sanz C, Rivas C, Benitez J. Correlation between mutations in p53 gene and protein expression in human lymphomas. *Am J Hematol* 1997;55:1-8.
36. Villuendas R, Piris MA, Algara P, Sanchez-Baeto M, Sanchez-Verde L, Martinez JC, Orradre JL, Garcia P, Lopez C, Martinez P. The expression of p53 protein in non-Hodgkin's lymphomas is not always dependent on p53 gene mutations. *Blood* 1993;82:3151-3156.

Chapter 10

General discussion

General discussion

Insight in the pathogenesis of gastrointestinal lymphomas, and in particular in that of gastric lymphomas has greatly increased in the last 15 years. The recognition that gastric lymphomas may develop from mucosa-associated lymphoid tissue acquired in response to a chronic active *H.pylori* gastritis elucidated the paradoxical finding of lymphomas in an organ which is normally devoid of lymphoid tissue.

However, many questions remain, most of them centred around the indolent behaviour of MALT lymphoma. In the present chapter we will discuss three items directly and indirectly related to this question. First, the cell of origin of MALT lymphoma, second the relation between small and large cell lymphoma and third the possible infectious origin of the lymphoma.

When Isaacson proposed his MALT concept in 1983 he described the lymphoma cells as centrocyte-like cells¹. It was hypothesized that these lymphoma cells were 'homing' lymphocytes, i.e. lymphocytes that do recirculate, but home to the mucosal tissues. Nowadays, these lymphomas of mucosa-associated lymphoid tissue are thought to originate from memory B-cells residing in the marginal zones of mucosal lymphoid tissues² Marginal zone lymphocytes have been shown to be non-circulating lymphocytes in rodents³, but their exact role in humans is not clear. However, recent investigations showed a preferential expression of both the human mucosal addressin cell adhesion molecule 1 (MadCAM-1)⁴ and its exclusive mucosal homing integrin receptor $\alpha 4\beta 7$ ⁵ in gastrointestinal mucosal lymphoid tissue and lymphoma. These findings underscore the distinctive character of mucosal lymphomas and may also form an explanation for their characteristic dissemination pattern and better prognosis which was also found in the present study (chapter 7).

Large cell primary gastric lymphomas are much more common than small cell lymphomas of MALT. Unless a small cell component displaying the features of lymphoma of mucosa-associated lymphoid tissue is present in biopsy or resection material, large cell lymphoma of MALT cannot be distinguished from other large cell lymphomas. Combining this experience with the results that large cell lymphomas with a MALT component had a better prognosis (chapter 7), it is obvious that extensive sampling during endoscopy is necessary. The relationship between small and large cell gastric (MALT) lymphoma remains controversial. Several studies could demonstrate a clonal link between small and large cell gastric lymphoma⁶⁻⁸. In the present thesis (chapter 6) we found numerical aberrations of chromosome 12, 15 and 18 in both small and large cell lymphoma. In contrast to observations described in the literature⁹, we could not confirm the frequent presence of trisomy of chromosome 3 in primary gastric lymphoma, neither in the small cell nor in large cell cases. However, the fact that large cell mucosal

lymphomas do not express $\alpha 4\beta 7$ in contrast to small cell MALT lymphomas is an argument against a direct relationship⁵. From these observations it is at least conceivable that large cell gastric lymphoma is a heterogeneous condition. This may also be concluded from chapter 3, in which large cell lymphomas displayed a wide range of proliferative and apoptotic activity. Unfortunately, this variety in cell turnover was not reflected in clinical differences between these lymphomas (chapter 7). Future studies should be performed to identify prognostic parameters in this large cell lymphoma subgroup.

If small cell lymphomas indeed progress to large cell gastric lymphoma, there is probably one point at which the lymphoma is no longer dependent on the antigenic drive provided by *H.pylori*. Since this point of no return probably not exactly corresponds to the point of transition of small to large cell lymphoma, it is conceivable that already a proportion of the small cell lymphomas are autonomously growing and consequently are no longer responsive to *H.pylori* eradication. Few studies have investigated the presence of *H.pylori* in large cell lymphomas, which may be a first indication of the role of this bacterium in this type of lymphoma.

In this thesis *H.pylori* was present in both histological grades of lymphoma, but more frequent in small cell lymphomas (chapter 4). In contrast to other studies, detecting *H.pylori* in over 90% of the cases we found a percentage not exceeding that in the normal population. Bacterial flora, other than *H.pylori*, was also frequently detected, especially in large cell lymphoma (chapter 5). Several explanations are possible for the presence of these bacteria, including colonization as a result of decreased gastric acid secretion either due to atrophic gastritis or to the tumour itself. Furthermore, these patients often had gastrointestinal complaints before gastric lymphoma was diagnosed and may have been treated with gastric acid inhibitors, resulting in bacterial overgrowth. Instead of being innocent bystanders, these bacteria might also be related to the development of gastric lymphoma, either by providing an antigenic stimulus or by the production of toxic metabolites.

The antigen-driven (or infectious ?) nature of this lymphoma is one of the most intriguing topics of investigation^{10,11}. The history of gastric lymphoma is filled with questions about its malignancy, probably best illustrated by the term pseudolymphoma, used in the past¹²⁻¹⁴. However, prove for the malignant nature of gastric lymphoma has definitely been found, for example in its monoclonal nature, their invasive properties and the ability to disseminate to and beyond regional lymph nodes.

Probably, gastritis and lymphoma are two conditions with a continuous spectrum of transitional conditions in between. Some factors regulating this process have

already been determined, but the major issue, the point at which gastric lymphomas start autonomous, antigen-independent growth, is still not exactly known.

Confusing is the observation that also in proven monoclonal lymphoma, remissions have been established after eradication of *Helicobacter pylori*¹⁵. It should be realized however that such monoclonal lymphomas might in fact be oligoclonal, and PCR only picked up one clone. If true however, this may support the vision that gastric lymphoma arises from a polyclonal (antigen-dependent) proliferation, through an oligoclonal stage into a monoclonal stage, in which the oligoclonal stage may react to eradication therapy, whereas true monoclonal disease does not.

Probably, this question will be one of the major topics in future studies of gastric lymphoma. Moreover, this antigen related tumour growth may be useful for the study of other malignancies. In particular, intestinal lymphomas (in part also antigen driven) which differ from gastric lymphomas, in that they are derived from already present gut associated lymphoid tissue, whereas gastric lymphomas arise from acquired mucosa associated lymphoid tissue, may be studied according to this model. Regression of duodenal and rectal non-Hodgkin's lymphoma of MALT type have already been noticed in response to *H.pylori* eradication^{16,17}.

In summary, the transitions between the phases recognized in mucosa-associated lymphoma (MALT/gastritis, small cell lymphoma and large cell lymphoma) require further research. The nature of the antigenic drive present in the first two phases has to be further elucidated and may provide a target for modification of the deranged immune reaction that seems to be present. Maybe the role of intratumoral T-cells is essential here. It is likely that in the overt lymphoma phase the response to antigen is lost. Factors marking this transition, like the accumulation of certain genetic abnormalities, would be useful in planning therapy of mucosa-associated lymphoid tissue lymphoma.

References

1. Isaacson P, Wright DH. Malignant lymphoma of mucosa-associated lymphoid tissue. *Cancer* 1983;52:1410-1416.
2. Harris NL, Jaffe ES, Stein H, Banks PM, Chan JKC, Cleary ML, Delsol G, De Wolf-Peeters C, Falini B, Gatter KC, Grogan TM, Isaacson PG, Knowles DM, Mason DY, Muller-Hermelink HK, Pileri SA, Piris MA, Ralfkiaer E, Warnke, RA. A revised European-American Classification of Lymphoid Neoplasms: A proposal from the international lymphoma study group. *Blood* 1994;84:1361-1392.
3. MacLennan ICM, Gray D, Kumararatne DS, Bazin H. The lymphocytes of splenic marginal zones: a distinct B-cell lineage. *Immunol Today* 1982;3:305-307.
4. Briskin M, Winsor-Hines D, Shyjan A, Cochran N, Bloom S, Wilson J, McEvoy LM, Butcher EC, Kassam N, Mackay CR, Newman W, Ringler DJ. Human mucosal addressin cell adhesion molecule-1 is preferentially expressed in intestinal tract and associated lymphoid tissue. *Am J Pathol* 1997;151:97-110.
5. Drillenburt P, VanDerVoort R, Koopman G, Dragosics B, van Krieken JHJM, Kluin Ph, Meenan J, Lazarovits AI, Radaszkiewicz T, Pals ST. Preferential expression of the mucosal homing receptor integrin $\alpha 4\beta 7$ in gastrointestinal non-Hodgkin's lymphomas. *Am J Pathol* 1997;150:919-927.
6. Montalban C, Manzanal A, Castrillo JM, Escribano L, Bellas C. Low grade gastric B-cell MALT lymphoma progressing into high grade lymphoma. Clonal identity of the two stages of the tumour, unusual bone involvement and leukaemic dissemination. *Histopathology* 1995;27:89-91.
7. Peng H, Du M, Diss TC, Isaacson PG, Pan L. Genetic evidence for a clonal link between low and high-grade components in gastric MALT B-cell lymphoma. *Histopathology* 1997;30:425-429.
8. Chan JKC, Ng CS, Isaacson PG. Relationship between high-grade lymphoma and low-grade B-cell mucosa-associated lymphoid tissue lymphoma (MALToma) of the stomach. *Am J Pathol* 1990;136:1153-1163.
9. Wotherspoon AC, Finn TM, Isaacson PG. Trisomy 3 in low-grade B-cell lymphomas of mucosa-associated lymphoid tissue. *Blood* 1995;85:2000-2004.
10. Isaacson PG, Spencer J. Is gastric lymphoma an infectious disease ? *Hum Pathol* 1994;24:569-570.
11. Eidt S, Bayerdörffer E, Stolte M. Treat the infection and cure the cancer. *Lancet* 1995;345:874-875.
12. Berry GR, Mathews WH. Gastric lymphosarcoma and pseudolymphoma: Reappraisal of 12 cases of gastric lymphosarcoma. *Can Med Assoc J* 1967;96:1312-1316.
13. Highman LM, Fantelli FJ, Hermann RE. Pseudolymphoma of the stomach. *Arch Surg* 1981;116:227-229.
14. Stroehlein JR, Weiland LH, Hoffman HN, Judd ES. Untreated gastric pseudolymphoma. *Digestive Diseases* 1977;22:465-470.

15. Savio A, Franzin G, Wotherspoon AC, Zamboni G, Negrini R, Buffoli F, Diss TC, Pan L, Isaacson PG. Diagnosis and posttreatment follow-up of *Helicobacter pylori*-positive gastric lymphoma of mucosa-associated lymphoid tissue: histology, polymerase chain reaction, or both ? Blood 1996;87:1255-1260.
16. Nagashima R, Takeda H, Maeda K, Ohno S, Takahashi T. Regression of duodenal mucosa-associated lymphoid tissue lymphoma after eradication of *Helicobacter pylori*. Gastroenterology 1996;111:1674-1678.
17. Matsumoto T, Iida M, Shimizu M. Regression of mucosa-associated lymphoid-tissue lymphoma of rectum after eradication of *Helicobacter pylori*. Lancet 1997;350:115-116.

Summary

Summary

Primary gastrointestinal non-Hodgkin's lymphoma is an uncommon disease which is gradually emerging as a specific clinical and pathological entity. Its interesting pathogenesis has partly been elucidated in the last decade, but many questions remain. These concern especially its classification, clinical staging and indolent behaviour. In this thesis, therefore, both clinical and pathological features of gastrointestinal lymphomas were studied in relation to these issues.

In **chapter 1**, a historical overview on the subject of gastrointestinal lymphoma is given. Important hallmarks are the definition of the MALT concept and the relationship of *Helicobacter pylori* with this disease. Also, the evolution of pathological classifications and clinical staging classifications is described. Finally, the aims of the present thesis are discussed.

The study population has been selected from a population-based registry. In the Limburg Working Party on lymphoma every new lymphoma case in a region of about a million inhabitants is discussed and registered. **Chapter 2** describes the inclusion criteria and provides some general information on the cohort studied (age, sex, stage, localisation in the gastro-intestinal tract). Background information is given on the techniques used in this thesis.

Gastric lymphomas account for the majority of gastrointestinal NHL. In the past gastric and intestinal lymphomas were studied together, but it has become clear that they have a different pathogenesis. Gastric lymphoma is an rather indolent tumour, and in studying its growth pattern we wanted to shed some light on its pathogenesis. Tumour growth is nowadays considered to depend on both cell proliferation, as well as cell death. In **chapter 3** these two features were studied. It appeared that small cell gastric lymphomas grow mainly by a prolonged survival possibly as a result of the inhibition of apoptosis by bcl-2, whereas large cell gastric lymphomas expand largely by rapid proliferation, despite the presence of higher apoptotic rates as well.

A series of events have taken place prior to autonomous growth in a small cell gastric lymphoma already. Mucosa associated lymphoid tissue seems to be a necessary prerequisite for the development of gastric lymphoma, as the normal stomach is devoid of any lymphoid tissue. Chronic active *Helicobacter pylori* infection is held responsible for the development of MALT in the stomach. Although the presence of *H. pylori* has been shown in a number of studies, few studies used specific techniques to investigate the presence of *H.pylori*, and even fewer examined their presence in large cell lymphomas. In this series *H.pylori* was present in both histological grades of lymphoma, but more frequently so in small cell lymphomas, as described in **chapter 4**. Also, a lower frequency of *H.pylori* was observed in our lymphoma cases, compared to other investigations. From our data we concluded that although *H.pylori* colonisation is an important factor in the

genesis of gastric lymphoma, other factors are also necessary. This holds especially true for large cell lymphomas, in which less *H.pylori* was found. However, using the specific method for the detection of *H.pylori*, it was noticed that apart from *H.pylori*, other bacteria were frequently seen, especially in the large cell cases.

Therefore, a more exact analysis of these bacteria was performed in **chapter 5**. Bacterial flora, other than *H.pylori*, was detected in a surprisingly high proportion of patients with gastric lymphoma. The possible explanations for this phenomenon are discussed in **chapter 5** and include among others decreased acid secretion by tumour tissue or bacterial overgrowth due to treatment for gastric complaints. Furthermore, the localisation and density of bacterial flora was compared in small and large cell lymphomas. Large cell lymphomas had a higher density of *H.pylori* in corpus mucosa. The role of other bacteria in gastric lymphoma has to be further elucidated.

Deregulation of genes in cancer is often caused by chromosomal abnormalities, and especially in non-Hodgkin's lymphoma specific numerical and structural chromosomal aberrations can be recognized in different subtypes. Sometimes, these specific abnormalities are even used for diagnostic purposes, and incorporation of genetic information in the REAL classification for lymphomas also shows their significance. Small cell gastric lymphomas of MALT have been studied for chromosomal aberrations by both classical cytogenetics as well as by interphase cytogenetics. Trisomy of chromosome 3 has been reported in a number of studies and is included as characteristic for small cell MALT lymphomas in the REAL classification.

Assuming that large cell primary gastric lymphomas are derived from small cell gastric MALT lymphoma, it could be expected that large cell lymphomas displayed the same aberrations than small cell lymphomas. Indeed, in **chapter 6** we describe numerical aberrations of chromosome 12, 15 and 18 in both small and large cell lymphoma. Also, more, and more different aberrations per case in large cell lymphomas were noticed. This may be explained by the accumulation of genetic abnormalities when tumours transform to more aggressive states and are genetically more instable.

In contrast to observations described in the literature, in this series we could not confirm the frequent presence of trisomy of chromosome 3 in primary gastric lymphoma, neither in the small cell nor in large cell cases. This discrepancy may, in part, be explained by the low number of cases in our study, however, taking into account the relative frequent trisomy described in literature, it would be very unlikely that in this series no small cell lymphoma bearing this abnormality would be present. Another explanation could be of technical nature. The probes used for the detection of chromosome 3 (D3Z1 and pAE0.68) both easily gave rise to minor binding sites, forcing us to apply rather stringent washes. This sometimes resulted in weak signals and it was difficult to find the right balance between stringent

washes and amplification of the signals. Misinterpretation of minor binding sites as signals, therefore, has to be considered a significant confounding problem in the evaluation of chromosome 3.

In **chapter 7**, a combined evaluation of both clinical and pathological data in gastric lymphoma is reported. Sixty-nine patients were studied retrospectively. Common complaints at presentation were abdominal pain, dyspepsia and weight loss. Overall five year survival was 46%. A better survival was found in patients with small cell lymphoma, MALT histology, low stage at presentation, young age and surgical therapy. Discussion exists about the interpretation of the role of MALT histology and surgical therapy. It seems that these factors are interrelated, because lymphomas with MALT histology were more often localized at presentation and as such more liable to surgical treatment.

Differences in apoptotic or proliferative indices did not identify prognostic significant subgroups. The clinical role of *H.pylori* or chromosomal aberrations could not be evaluated because of the low number of cases.

Chapters 8 and 9 describe cell turnover studies and clinical studies on primary intestinal lymphoma, respectively. A very interesting difference in the comparison between gastric and intestinal lymphoma is the notion that intestinal lymphomas are probably derived from already present gut associated lymphoid tissue, whereas gastric lymphomas are derived from acquired mucosa-associated lymphoid tissue. Furthermore, although MALT is abundantly present in the intestines and is generally not present in the stomach, gastric lymphoma is more common than intestinal lymphoma.

In this series we selected intestinal lymphomas associated to the mucosa and classified them in the same way as the stomach lymphomas. Furthermore, a refined histological classification was applied, in which the percentage of diffusely intermingled blasts was scored. As in gastric lymphoma, large cell intestinal lymphomas were more common than small cell lymphomas.

Proliferative and apoptotic indices were determined (**chapter 8**), and like in gastric lymphoma, were correlated with histology, small cell lymphomas showing a low cell turnover, whereas large cell lymphomas demonstrated high apoptotic and proliferative indices.

Comparing these cell turnover parameters in intestinal lymphoma to those in gastric lymphoma, it was noticed that apoptotic indices in intestinal lymphomas in general were slightly higher than in gastric lymphomas. This can be explained by the presence of T-cell lymphomas, which showed higher apoptotic indices. This higher rate of apoptosis may be partly explained by the relative absence of bcl-2 expression in T-cell lymphomas, but is probably also due to other factors involved in the regulation of apoptosis.

Overexpression of the p53 protein was not often present in intestinal lymphoma, and like in gastric lymphoma, was more common in large cell lymphomas as

compared to small cell lymphomas, suggesting a similar role in the progression of small to large cell lymphoma.

Chapter 9 shows that although only few patients had lymphomas overexpressing this p53 protein, these patients had a significantly worse survival. In general, also a higher proliferation was found in p53 positive lymphomas.

A more useful indicator of survival was the histological classification dividing cases in small and large cell lymphoma. In this series, none of the patients with small cell lymphoma died of the disease. Apart from histology, the B- or T-cell phenotype of the lymphoma was important. Although not significant, T-cell lymphomas were associated with a very poor survival.

There was no role for proliferation as a predictor of survival, however, apoptosis seemed to be of relevance. Patients with lymphomas displaying high apoptotic rates demonstrated a worse survival. The explanation for this phenomenon could be that most lymphomas with high apoptotic rates were large cell lymphomas.

In the general discussion (**chapter 10**), the results of this thesis are discussed in the context of the literature. Furthermore, interesting topics of future research are identified, in particular the antigen driven nature in these tumours and possibly also other tumours.

Samenvatting

Samenvatting

Het primaire non-Hodgkin lymfoom van het maag-darm kanaal is een zeldzame aandoening, die steeds meer gezien wordt als een specifieke klinische en pathologische entiteit. In de laatste tien jaar is veel van de ontstaanswijze van deze ziekte ontrafeld. Er blijven echter nog veel vragen over. Deze betreffen het relatief gunstige beloop van het maaglymfoom, en de classificatie en klinische stadiëring van deze ziekte. In dit proefschrift werden daarom klinische en pathologische kenmerken van maag-darm lymfomen bestudeerd in relatie tot deze aspecten.

In **hoofdstuk 1** worden de ontwikkelingen op het gebied van maag-darm lymfomen in chronologische volgorde beschreven. Mijlpalen waren de definiëring van het MALT concept (mucosa-geassocieerde lymfoïde weefsel) en de betrokkenheid van het micro-organisme *Helicobacter pylori* in deze ziekte. Ook worden de ontwikkelingen in histologische classificaties en klinische stadiëringen beschreven. Uiteindelijk worden de doelstellingen van dit onderzoek besproken.

Het bestudeerde cohort werd geselecteerd uit de archieven van het Limburgs Lymfomen Panel, dat maandelijks elk nieuwe lymfoproliferatieve aandoening bespreekt die wordt gediagnostiseerd in deze regio, die ongeveer 1 miljoen mensen beslaat. In **hoofdstuk 2** worden de inclusiecriteria beschreven en daarnaast algemene informatie omtrent de leeftijdsverdeling, man-vrouw ratio, stadium en lokalisatie in het maag-darm kanaal van het geselecteerde cohort. Achtergrond-informatie omtrent de gebruikte methoden om celgroei, geprogrammeerde celdood en numerieke chromosoomafwijkingen te meten wordt ook beschreven in dit hoofdstuk.

De meeste maag-darm lymfomen ontstaan in de maag. Alhoewel maag- en darmlymfomen in het verleden samen bestudeerd werden, is het nu duidelijk dat zij een volstrekt andere ontstaanswijze en klinisch beloop hebben. Het primaire maaglymfoom is een aandoening met een relatief gunstig beloop. Middels de bestudering van het groeipatroon van deze aandoening hebben we geprobeerd iets van de pathogenese op te helderen. In **hoofdstuk 3** werden zowel de mate van celdeling als de mate van celdood in de vorm van apoptose gemeten, zodat een indruk van celaanmaak en celafbraak werd verkregen. Dit omdat de laatste jaren steeds duidelijker wordt dat een verstoring in de weefselhomeostase zowel een gevolg kan zijn van verstoorde aanmaak als van verstoorde afbraak. Uit de data kwam duidelijk naar voren dat kleincellige maaglymfomen vooral groeien als een gevolg van een verstoorde celdood (apoptose), mogelijk ten gevolge van inhibitie van dit proces door hoge expressie van het bcl-2 eiwit in deze tumoren. Dit in tegenstelling tot grootcellige maaglymfomen, die een zeer snelle turnover van cellen liet zien, met zowel snelle aanmaak als ook snelle afbraak van cellen.

Voordat een kleincellig maaglymfoom zich autonoom kan ontwikkelen, hebben al een aantal andere processen plaatsgevonden. De aanwezigheid van mucosa-

geassocieerd lymfoïd weefsel (MALT) lijkt noodzakelijk te zijn voor de ontwikkeling van een maaglymfoom, gezien het feit dat er in de normale maag geen georganiseerd lymfoïd weefsel aanwezig is. Chronisch actieve *Helicobacter pylori* gastritis lijkt verantwoordelijk voor de ontwikkeling van dit MALT. Ofschoon de aanwezigheid van deze bacterie is aangetoond in een aantal studies, is dit vrijwel nooit met specifieke methodes gedaan en daarnaast bijna alleen in kleincellige maaglymfomen. **Hoofdstuk 4** beschrijft de resultaten van deze studie, waarin *Helicobacter pylori* werd gevonden in zowel kleincellige als grootcellige maaglymfomen, maar vaker in de eerstgenoemde. Bovendien lieten deze data, in vergelijking met de bestaande literatuur, een lagere frequentie van *Helicobacter pylori* aanwezigheid zien. Hieruit kan geconcludeerd worden dat *Helicobacter pylori* kolonisatie van de maag zeker een belangrijke factor is in de ontwikkeling van het maaglymfoom, maar dat er daarbij ook andere factoren nodig zijn. Dit geldt met name voor de grootcellige maaglymfomen, waarin minder *Helicobacter pylori* werd gezien. Dankzij het gebruik van een specifieke detectiemethode voor *Helicobacter pylori*, werd echter ook opgemerkt dat in grootcellige lymfomen vaker andere bacteriële flora werd gezien. Daarom werd in **hoofdstuk 5** een aanvullende analyse verricht ten aanzien van deze non-*Helicobacter pylori* bacteriële flora. Deze werd in een verrassend hoog percentage van de grootcellige lymfomen gevonden. Mogelijke verklaringen voor dit fenomeen worden besproken en zijn onder andere een verminderde zuurproductie door de tumor en bacteriële overgroei tengevolge van behandeling voor dyspepsie. Ook werden de localisatie en densiteit van bacteriële flora vergeleken in klein- en grootcellige lymfomen. Hierbij werden nauwelijks verschillen gevonden. De rol van deze andere bacteriële flora dient nog verder uitgezocht te worden, mogelijk dat ook deze bacteriën een rol spelen in de pathogenese van deze ziekte.

Bij tumorgroei worden vaak genetische afwijkingen in de zin van chromosoom afwijkingen aangetroffen. Vooral non-Hodgkin lymfomen zijn een voorbeeld van tumoren die vaak specifieke structurele chromosomale afwijkingen vertonen in de verschillende subtypes. Deze specifieke afwijkingen worden zelfs gebruikt voor diagnostische doeleinden. Verder blijkt hun belang ook uit de inclusie van genetische informatie in de REAL (Revised European-American Lymphoma) classificatie. Kleincellige MALT lymfomen zijn op genetisch niveau bestudeerd, met behulp van klassieke cytogenetica en in situ hybridisatie. Een trisomie (drie chromosomen van één type in plaats van twee) van chromosoom drie is beschreven in enkele studies en wordt in de REAL classificatie genoemd als karakteristiek voor deze lymfomen.

Als grootcellige maaglymfomen inderdaad voortkomen uit kleincellige primaire maaglymfomen, zouden ze in ieder geval dezelfde genetische afwijkingen moeten hebben als kleincellige lymfomen. Inderdaad worden in **hoofdstuk 6** numerieke afwijkingen van chromosoom 12, 15 en 18 beschreven in beide histologische

entiteiten. Daarnaast werden in grootcellige lymfomen vaker afwijkingen gezien en ook vaker meer afwijkende chromosomen per lymfoom. Een mogelijke verklaring hiervoor is de toename van genetische instabiliteit in meer agressieve grootcellige lymfomen met een snellere turnover.

De in de literatuur beschreven frequentie van voorkomen van trisomie 3 kon niet worden bevestigd in onze studie. Mogelijk is dit een gevolg van het lage aantal bestudeerde lymfomen, echter gezien de frequentie beschreven in de literatuur is het zeer onwaarschijnlijk dat in deze serie zo weinig trisomieën van chromosoom drie werden gezien. Een andere verklaring zou een technische kunnen zijn. De gebruikte probes voor de detectie van chromosoom drie gaven makkelijk aanleiding tot aspecifieke aanhechting, wat zeer grondig wassen noodzakelijk maakte. Dit laatste resulteerde weer in zwakkere signalen, en het bleek moeilijk een goed evenwicht te vinden tussen de wasprocedure en signaalintensiteit. Misinterpretatie van signalen lijkt daarom een moeilijk probleem te zijn bij de evaluatie van chromosoom drie.

In **hoofdstuk 7** worden de resultaten beschreven van een gecombineerde analyse van klinische en pathologische data. In deze studie werden 69 patiënten retrospectief bestudeerd. Frequente klachten en symptomen bij presentatie waren buikpijn, dyspepsie en gewichtsverlies. De overall vijf-jaars overleving was 46%. Een betere overleving werd gevonden in patiënten met kleincellige lymfomen, MALT histologie, presentatie in lage stadia, jongere leeftijd en chirurgische therapie. Over de rol van MALT histologie en chirurgie is enige discussie mogelijk. Deze twee prognostisch gunstige factoren kunnen aan elkaar gerelateerd zijn, omdat lymfomen met MALT histologie vaker in een lager stadium bij patiënten vastgesteld worden. Dit maakt deze lymfomen toegankelijker voor chirurgische therapie. Groepen ingedeeld volgens lage of hoge celdelingsgraad of celdood ratio lieten geen prognostische verschillen zien. De klinische rol van *Helicobacter pylori* of chromosoom afwijkingen kon niet geëvalueerd worden in verband met het lage aantal gevallen, waarin deze resultaten bekend waren.

Cel turnover en retrospectieve klinische analyse in primaire darmlymfomen worden respectievelijk in **hoofdstuk 8 en 9** beschreven. Een interessant verschil tussen maag- en darmlymfomen is het feit dat darmlymfomen waarschijnlijk voortkomen uit al aanwezig lymfoid weefsel, terwijl dit in de maag eerst verworven moet worden. Daarnaast leidt de aanwezigheid van mucosa-geassocieerd weefsel in de darm klaarblijkelijk veel minder vaak tot de ontwikkeling van een lymfoom, aangezien ongeveer 2/3 van alle maag-darm lymfomen zich in de maag manifesteert.

In deze studie werden darmlymfomen geselecteerd die een duidelijke relatie met de mucosa hadden en deze werden op dezelfde manier geclassificeerd als de maaglymfomen. Bovendien werd een verfijnde histologische classificatie gebruikt, waarin het percentage blasten werd gescoord. Net zoals in maaglymfomen, werden

ook in de darmlymfomen meer grootcellige lymfomen gevonden.

Celdeling en celdood ratios werden bepaald en bleken ook hier geassocieerd met het histologisch patroon (**Hoofdstuk 8**). Ook in darmlymfomen lieten kleincellige varianten een veel lagere cel turnover ratio zien dan grootcellige lymfomen.

Vergelijking met de waarden in maaglymfomen, toonde dat celdood ratio's in darmlymfomen over het algemeen genomen hoger waren. Dit zou kunnen worden verklaard met de aanwezigheid van T-cel lymfomen, die veel celdood lieten zien. Dit laatste zou een gevolg kunnen zijn van de afwezigheid van bcl-2 expressie in T-cel lymfomen, maar is waarschijnlijk ook een gevolg van andere factoren die een rol spelen in de regulatie van celdood.

Overexpressie van het p53 eiwit kwam niet vaak voor in darmlymfomen, en was net zoals in maaglymfomen vaker aanwezig in grootcellige lymfomen. Dit suggereert in beide een rol in de progressie van klein- naar grootcellige lymfomen, zoals die ook in nodale lymfomen wordt beschreven.

Alhoewel p53 slechts zelden voorkwam, hadden patiënten met een p53 positief lymfoom een significant slechtere prognose (**Hoofdstuk 9**). In het algemeen werd ook een hogere proliferatie gevonden in p53 positieve lymfomen. Een meer bruikbare indicator voor de prognose was de histologische classificatie in klein en grootcellige lymfomen. In de bestudeerde groep was er geen enkele patiënt met een kleincellig darmlymfoom die overleed tengevolge van deze ziekte.

Afgezien van de histologie was ook het B- of T-cel fenotype belangrijk. Ofschoon niet significant, leken T-cel lymfomen een duidelijk slechtere prognose te hebben.

Ook in darmlymfomen was celgroei geen voorspeller van de survival, celdood echter wel van belang te zijn. Patiënten met een hogere celdood index leken een slechtere overleving te hebben. Een mogelijke verklaring hiervoor is dat dit ook met name de grootcellige lymfomen waren.

In **hoofdstuk 10** worden de resultaten van deze studie beschreven in de context van bestaande literatuur. Daarnaast worden een aantal interessante aspecten van deze zeldzame ziekte naar voren gebracht die nader onderzoek behoeven.

Appendix

Figure 3.1 Proliferation Index (PI) measured by Mib-1 expression in small (Fig. 1A) and large (Fig. 1B) cell lymphoma, bcl-2 immunoreactivity in small (Fig. 1C) and large (Fig. 1D) cell and Apoptotic Index (AI) measured by TUNEL expression in small (Fig. 1E) and large (Fig. 1F, arrows indicating TUNEL positive cells showing morphological criteria of apoptosis) cell primary gastric lymphoma. Original magnification 112x.

Figure 4.1 The presence of *Helicobacter pylori* (arrows) demonstrated in MG (non-specific) and IHC (specific) stainings in a small cell primary gastric lymphoma of mucosa associated lymphoid tissue. A: modified Giemsa, B: immunohistochemistry, original magnification 312x.

Figure 4.2 Immunohistochemical staining of a small cell primary gastric lymphoma of mucosa-associated lymphoid tissue, in which *Helicobacter pylori* is seen deep in the gastric pits, at the level of the neck of the gastric glands (arrows). In this case, *Helicobacter pylori* was not seen in or on the mucus layer. Original magnification 312x.

Figure 6.1

In situ hybridisation results in different lymphoma cases. A) 91-5995 showing normal (disomic) nuclei for chromosome 1, B) 92-2181 showing a trisomy for chromosome 1, C) 91-2958 showing nuclei with three and four spots for chromosome 18, D) 82-22923: trisomy for chromosome 12, E and F showing case nr 86-10044 with a trisomy for chromosome three (F using the CARD system). Inlay demonstrates an example of nuclei with lots of minor binding spots, complicating the evaluation.

Figure 8.1
A Small B-cell lymphoma of MALT immunohistochemically stained with the mib-1 antibody. The germinal center shows a very high proliferation index, in contrast to the lymphoma itself. *Inset* shows a more diffuse part of the same lymphoma.
B Mib-1 immunoreactivity in a large B-cell lymphoma, showing a high proliferation index.
C Apoptotic index using the TUNEL-assay in a large B-cell lymphoma of MALT. Morphological features of apoptosis (chromatin beaded against the nucleus) can be detected in a few TUNEL positive cells (arrows). *Seale bar* 20 μ m.

Dankwoord

Dankwoord

In juni 1992 kwam ik als 3e jaars studente geneeskunde voor mijn wetenschapsstage terecht bij de afdeling Pathologie. Dat ik inmiddels zes jaar later het dankwoord van dit proefschrift mag schrijven had ik nooit kunnen bedenken. Misschien een goed moment om terug te kijken, alvorens een aantal mensen apart te bedanken.

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Curriculum Vitae

Curriculum Vitae

Ingrid AM Gisbertz

De schrijfster van dit proefschrift werd geboren in Sittard op 7 maart 1970. In 1988 behaalde zij het VWO diploma en begon in hetzelfde jaar aan de studie Gezondheidswetenschappen aan de Universiteit Maastricht. Een jaar later begon zij aan de studie Geneeskunde, aan dezelfde Universiteit, die in 1997 werd afgesloten met het Artsexamen. Het in dit proefschrift beschreven onderzoek werd grotendeels uitgevoerd tijdens de studie geneeskunde. Van september 1995 tot en met augustus 1996 werd deze studie onderbroken voor een aanstelling als onderzoeker bij de vakgroepen Interne Geneeskunde en Pathologie van het Academisch Ziekenhuis Maastricht. Vanaf september 1997 is Ingrid Gisbertz werkzaam als assistent interne geneeskunde in het Diaconessenhuis te Eindhoven. Op 1 april 1998 is zij gestart met de opleiding Interne Geneeskunde in hetzelfde ziekenhuis (opleider Dr HR Haak).