UNIVERSITA' DEGLI STUDI DI MILANO

FACULTY OF MEDICINE AND SURGERY

RESEARCH DOCTORATE IN GASTROENTEROLOGY

CICLE XXVII

PERSONALIZED MANAGEMENT OF CELIAC DISEASE: RISK STRATIFICATION AND NOVEL STRATEGIES FOR COMPLICATED PATIENTS

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Dissertation for the Degree

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R09538

Academic Year 2014

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SUMMARY

Background. Celiac disease is a widespread autoimmune disease diagnosed at increasing frequency. Celiac patients regularly attend medical services and present a benign course. The knowledge of clinical and biological factors predictive of malignant evolution is poor. Thus, management of such a large population of patients still lacks evidence-based criteria in order to target diagnostic strategies to the subgroup ”at-risk” to develop small bowel complications.

Aim. To identify celiac patients’ characteristics associated to a higher probability of complicated course and to evaluate the role of available screening and diagnostic approaches and coming biomarkers for the early diagnosis of small bowel malignancies in celiac disease.

Methods. A population-based registry (Varese province) was used to estimate the risk of intestinal lymphomas in undiagnosed celiac subjects, taking into consideration different settings of celiac prevalence and relative risks for intestinal lymphomas in comparison with the incidence of the lymphomas in the population. Treated celiac patients were selected on the basis of clinical flags of non-responsiveness or lack of compliance to treatment for targeted small bowel endoscopic examinations, evaluating the diagnostic yield of capsule endoscopy and double-balloon enteroscopy in the recognition of intestinal tumors at an early stage. miRNA profiles in type I and II enteropathy-associated T cell lymphoma were explored to identify eventual celiac related signature to be applied to subgroups of patients with different levels of risk for stratification purpose.

Results. In the considered population (815,362 inhabitants), during a five years period, the relative risks of undiagnosed celiac disease for gastrointestinal B- and T-cell lymphomas ranged from 1.0 to 2.0 for 1:100 celiac disease prevalence. In the selected series of 105 celiac patients, the diagnostic yield of capsule endoscopy and double-balloon enteroscopy for malignancies was 2.9%. When compared to the registered population, the relative risk for adenocarcinoma and neuroendocrine tumors in the studied cohort was 410 (CI 95% 102-1640, $P<0.0001$). Double-balloon enteroscopy was useful to histologically characterize even minor lesions detected at capsule endoscopy. A single miRNA (under patent) resulted upregulated in type I enteropathy-associated T cell lymphoma with confirmation at qPCR ($p=0.0012$). When mucosa specimen were evaluated, miRNA profiling of refractory patients was highly homogeneous ($p=0.001$ vs both type I and II enteropathy-associated T cell lymphoma).

Conclusions. Undiagnosed/untreated celiac patients did not show a different risk of developing gastrointestinal lymphomas than the general population. Capsule endoscopy and double-balloon enteroscopy could be considered at an early stage in the diagnostic management of a subset of celiac patients at risk for small bowel malignancies. We expect to identify miRNA signatures as a novel biomarker predictive of disease aggressiveness to support the implementation of a personalized clinical approach, in order to optimize resources employment and, through a timely pharmacological intervention, improve prognosis of these subset of celiac patients.

1.CELIAC DISEASE AND SMALL BOWEL COMPLICATIONS

1.1 Celiac disease

Celiac disease (CD) is an autoimmune disorder precipitated, in genetically predisposed subjects, by a known environmental factor, that is the ingestion of gluten. While in the past it has been usually considered a malabsorption syndrome affecting the children, CD is now recognized as a systemic autoimmune disorder with possible onset at any age.

Gluten is the major storage protein of wheat and other cereals. Gliadin, the alcohol-soluble gluten fraction, and its undigested fractions are responsible for the immune response activation, which is mediated by both the innate and the adaptive immune systems.

Such peptides (as an α-gliadin portion made up of 33 amino acids) pass the intestinal epithelial barrier and in the lamina propria interact with antigen presenting cells HLA class II DQ2 or DQ8 restricted. Type II transglutaminase deamidates gliadin peptides, increasing their immunogenicity. The adaptive response is mediated by CD4+ T cells with consequent release of proinflammatory cytokines (mainly interferon-γ). Due to the innate immune response, enterocytes express IL-15 and NK-G2D intraepithelial lymphocytes exert cytotoxic effects on enterocytes. Moreover, crypt hyperplasia and villous atrophy develops a consequence of extracellular matrix proteinases activation.

HLA-DQ2 or HLA-DQ8 is necessary but not sufficient condition for the development of CD. HLA genes contributes for less than 50% to the genetic substrate of CD, with other non-HLA genes perhaps contributing to the disease. Others unknown environmental factors, such as viral infection, may be involved in the pathogenesis.

Prevalence of CD in western countries is about 1% in screening population studies. [1] The disease is recognized not only throughout Europe and in countries populated by persons of European ancestry but also in the Middle East, Asia, South America and North Africa. The world distribution of CD seems to have followed the mankind wheat consumption and the migratory flows, registering

an increased frequency in many developing countries. It is mainly due to the Westernization of the dietary habit, changes in wheat production and preparation, and also increased awareness of the disease.[2]

Clinical presentation of CD varies according to age. Malabsorption symptoms and growth retardation represent common onset in infants and children.

Among adults, two to three times as many women have the disease as men, probably reflecting the prevalence of autoimmune diseases. Mono-paucisymptomatic or silent presentation in adults include functional-like gastrointestinal symptoms, iron-deficiency anemia, unexplained osteoporosis or elevation of transaminases, recognition during surveillance among first-degree relatives of CD patients and subjects with known CD-associated conditons (i.e. Down syndrome, type 1 diabetes). Classic onset occurs in the elderly too. Substantial proportion of patients are overweight. Persons with celiac disease have an increased risk of autoimmune disorders as compared with the general population. [3] Patients often have symptoms for a long time and undergo multiple investigatons before CD is diagnosed.[4]

Diagnosis of CD relies on serology and duodenal biopsy, performed while the patient is on a gluten-containing diet. Biopsy remains the mainstay for the diagnosis in the adulthood. [5] To state definite diagnosis of CD, villous atrophy is required (grade $\geq$ 3, according to Marsh-Oberhuber [6]). However, lesser degrees of damage ($\geq$25 IELs but no villous atrophy) combined with positive serology may also represent CD. Serological detection depends on the presence of specific endomysial antibodies (EMAs), IgA anti-tissue transglutaminase antibodies (IgA-TG2) and/or deamidated antigliadin antibodies (DGP, either IgA or IgG isotype). IgG-TG2 is primarily useful in patients with known IgA deficiency. The prevalence of seronegative CD is 6–22% of all diagnosed cases. [7, 8] Biopsy of the duodenum for a diagnosis of CD should be performed irrespective of the prior performance of serological tests, if the patient exhibits symptoms or signs of CD, such as diarrhoea, weight loss or anaemia. [9]

1.2 Celiac disease and malignant complications

The association between CD and cancer is long established. Cases of small bowel (SB) lymphoma were described in the presence of clinical manifestation of malabsorption in the first half of the twentieth century.[10] In 1962, Gough firstly reported five cases of intestinal lymphoma in CD, hypothesizing a predisposing role of the enteropathy toward the malignant transformation.[11] Epithelial cancers of the gastrointestinal tract were incostantly reported in CD with higher frequency than expected and contributing to higher mortality rate in patients affected by CD.[12-14] Liver cancer has been isolatedly reported with a higher frequency in CD,[15] while incidence rate seemed to be reduced as far as breast cancer is concerned.[12, 16]

Mechanism for the development of malignancies in patients with CD is not known: however, hypothesis involving increased intestinal permeability of environmental carcinogens, chronic inflammation, chronic antigenic stimulation, circulation of proinflammatory cytokines, alterations in the immune surveillance and nutritional deficiencies have been proposed.

1.2.1 Enteropathy-associated T cell lymphoma and other lymphoproliferative disorders

Enteropathy-associated T cell lymphoma (EATL) is a rare non-Hodgkin intestinal lymphoma. Estimated annual incidence rate is 0.5-1 per million people in Western countries. [17, 18] Peak of incidence is evident in the sixth decade of life.

According to the WHO classification (2008), EATL is reported among the peripheral T cell neoplasms. Type I EATL has been described with either overt or clinically silent gluten-enteropathy and it is more spread in the European countries. Secondary EATL develops in adult patients previously diagnosed with CD, following a GFD who subsequently manifest exacerbation of symptoms. Onset of the complication most of times occurs during the first 5 to 10 years after CD diagnosis, but it has been reported in long-standing CD too. Primary EATL arises in subjects without a known history of CD. Serological tests may be helpful in identifying the associated

enteropathy even if antibodies may disappear at this stage. Type II EATL has a worldwide distribution, it is usually sporadic and it should be considered a separate entity.

EATL represents about 35% of intestinal lymphoma. Preferential site is the jejunum, but it may develop in the ileum, lymph nodes, stomach and colon. Up to 25% of cases presents a multifocal distribution. As far as immunophenotype is concerned, type I EATL is characterized by $CD3^{+}$,$CD4^{-}$, $CD8^{-}$,$CD5^{-}$, $CD7^{+}$,$CD103^{+}$,$CD56^{-}$,$TCR\beta^{+}$/ $^{-}$cells, mainly CD30 positive.

Systemic or B symptoms should be taken as alarm signs, although they are present in less than 30% of EATL.[19] Non response to GFD or new onset of diarrhea, abdominal pain and unexplained weight loss should raise the suspicion in patients on GFD. Recognition of primary EATL could be difficult due to low specificity of the clinical picture.

EATL may represent the end-stage of a refractory course of CD, occurring mainly in type II RCD. Some CD patients complicating directly into EATL have been described,[20] while EATL has been described in only 2 patients with type I RCD.[21]

Early studies indicated a relative risk for lymphoma in CD of the order of 50-100 but studies were conducted in tertiary referral centers and included patients with concomitant diagnosis of CD and lymphoma, introducing relevant bias as far as diagnostic ascertainment is concerned.[22-24] More recently, relative risk for gut lymphoma in CD has been estimated to be about 17 folds the risk of the general population as from case controls studies [25, 26] and up to 40 folds, when the association was evaluated in a prospective population-based study: Card found such a risk in the celiac population attending a secondary referral center with regular follow up, perhaps allowing an accurate detection of complication than in data obtained from population registry. Of note, malignant cases detected in the CD peri-diagnosis period were not included and patients were younger than in first studies.[16]

An increased risk for non-Hodgkin lymphoma (NHL) was attributed to CD patients too, as from Ema screening of newly diagnosed NHL in a case-control study, being estimated in about 3-folds.[25] Population-based studies showed standardized incidence ratio between 6 and 12 [15, 16, 27] and absolute rates of 8-13/10000 persons year.[16, 27, 28] Some limits may derive from studies having discharge diagnosis as inclusion criteria and therefore evaluating perhaps more severe inpatients. [15, 29, 30] Increased risk for NHL including both T-cell and B-cell types and occurring in both gastrointestinal and extraintestinal sites was reported. [30, 31]

Prognosis of EATL is very poor and this is particularly evident when it develops in association with type II RCD, with a 5-year survival rate between 8 and 20% among patients with both EATL and RCD II.[32]

A definite diagnostic algorithm in EATL has not been accepted yet. Computerized tomography or magnetic resonance enterography allow visualization of bowel wall thickening, intussusception, lymph nodes enlargement and cavitation, raising suspicion of RCD II or EATL. [33, 34] PET scan showed high sensivity but low specificity for the detection of EATL. [35] Imaging techniques have been integrated by the more recent introduction of enteroscopic tools (capsule endoscopy and device-assisted enteroscopy) for the visualization of SB mucosal alterations and histological characterization.

Evidence-based data on EATL treatment are lacking due to low incidence of the disease and frequently poor nutritional conditions of the affected patients. First-line surgery has a prominent role for those patients presenting with complications at the onset, that is with obstruction, bleeding or perforation. Moreover, surgical approach often precedes medical therapy, according to the higher risk of such complications during chemotherapy.

Standard-dose multidrug chemotherapy and, particularly, CHOP regimen represent the most common treatment choice for EATL, with the achievement of an overall 5-year survival of 9-

22%.[19, 36] More promising results came from mainly restrospective, small sized studies exploring the role of high-dose chemotherapy followed by autologous stem cell transplantation (ASCT): CHOP has been traditionally adopted as first-line chemotherapy and BEAM (carmustine, etoposide, cytarabine and methotrexate) as conditioning regimen, respectively. The choice of a more intensive treatment should be based on an accurate selection of those patients who can tolerate it.

1.2.2 Small bowel adenocarcinoma

Malignant tumors of the small bowel comprise 3% of all gastrointestinal malignancies. Primary SB adenocarcinoma is the most common histological subtype, representing 35-50% of cases.

According to the EUROCARE data, the estimated number of annual new cases of SB adenocarcinoma in Europe is 3600, with an estimated incidence rate of 5.7 cases per million persons. [37]

Recognized settings for the development of SB adenocarcinoma are inflammatory bowel diseases (Crohn's disease, celiac disease) and genetic syndromes including familial adenomatous polyposis, hereditary non polyposis colon cancer (HNPCC), and Peutz-Jeghers syndrome. Consumption of red or smoked meat, saturated fat, refine carbohydrates, alcohol, obesity and smoking have been suggested among possibly related behavioral risk factors.

Presently, SB adenocarcinoma is reported to be the second most common malignancy (after lymphoma) in CD. One of the first descriptions reported a 8% prevalence of SB adenocarcinoma in CD population.[12] When SB adenocarcinomas were taken into account, a concomitant celiac enteropathy was observed in 13% of cases. [38]

Relative risks of developing SB adenocarcinoma in CD were reported between 10 and 70. [4, 12, 15, 31, 39] Some of the evidence comes from studies involving previous or concomitant diagnosis of malignancy and CD. [31] On the other hand, data showing a persistence of the risk of developing

intestinal adenocarcinoma during follow up period on GFD exist. [15, 40] The observation could support the idea that gluten avoidance could not prevent this complication. In line with this assumption, case reports highlights the chance of SB adenocarcinoma in patients with childhood onset and adequate treatment [41] and in case of histologically proven CD remission. [42] Moreover, low incidence of the tumor makes observations inconstant about the association between the malignancy and CD, with population-based cohort studies not reporting such an increased risk. [16, 43]

Most common site for SB adenocarcinoma is the duodenum (55-82%), followed by jejunum (11-25%) and ileum (7-17%); however, in celiacs it is more frequently located in the jejunum.

Pathogenic steps are not fully elucidated but an adenoma-carcinoma sequence hypothesis has been advanced. Microsatellite instability (MSI) has been documented in up to 60-70% of CD related intestinal adenocarcinoma [41, 44], locating CD among possible differential diagnosis when facing with SB adenocarcinoma with MSI. Aberrant methylation at CpG islands, an epigenetic phenomenon usually observed in tumors and other conditions like chronic inflammation, has been found in neoplastic (MINT and MGMT loci) and particularly (MLH1) in CD related neoplastic tissues, even if specificity is low. [41] APC mutation is less often observed than in colorectal cancer, even if an alteration of beta-catenin is probably involved. [45]

Diagnosis is usually delayed due to aspecific clinical presentation, including vague abdominal pain. Thus, occlusion and bleeding frequently characterize the onset of the disease at an advanced stage, when an emergent surgical intervention is needed.

Treatment approach is based on surgery and curative resection is achievable in about 40-65% of cases. Given its infrequency, no clear data are available about the role of chemotherapy and/or radiotherapy in the adjuvant phase and indications are extrapolated from the experience on colon cancer. Accordingly, French recommendation included a fluoropyrimidine and oxaliplatin-based

chemotherapy after a curative R0 resection of a stage III adenocarcinoma and, optionally, for pT4 stage II tumors (www.tncd.org). Five-year overall survival rate ranges from 14 to 33%, depending on tumor stage.

1.2.3 Risk factors for malignant complications

A small subset of the large celiac population actually develops malignant complications. The knowledge of the demographic, clinical, behavioral characteristics with prognostic implications is still incomplete. A profile of the celiac patient with a higher probability to show a lack of response to GFD or to present a malignancy during the lifetime is presently only partially understood. Attempts to identify such an "identikit" should be pursued in order to orient diagnosis and management of celiac patients.

Advanced age at CD diagnosis has been considered as one of the features possibly related to a different course of the disease. This can possibly be related to later introduction of GFD or, even more interestingly, to a different manifestation in the wide clinical spectrum of CD. In fact, in the elderly clinical symptoms leading to recognition of the enteropathy could also be more pronounced, with classical presentation being more represented than in the adulthood. Observation of such a relationship between age at diagnosis and complicated course, [46] however, has not always been confirmed.[47] The same work by Corrao et al. and other studies [48-50] outlined that overt clinical onset of the disease itself was more frequently associated with a complicated course than in case of paucisymptomatic clinical picture.

On the other hand, when risk of malignancy was evaluated in the pediatric celiac population, both increased [9, 39, 51] and not increased risks [15, 27, 52, 53] were described. With this regard, data should be taken into consideration looking at follow up time duration: particularly, lack of evidence of malignancy could be ascribed to the fact that young patients did not reach in the follow up time the age in which these kind of tumors mainly develop.

Again, a protective role of GFD has been questioned since conflicting data are present as far as neoplastic risk during follow up of treated patients is concerned. Askling et al. showed a risk reduction over treatment periods,[15] while risk appear quite stable in studies by Biagi and Gao et al.. [29, 46]

Similar considerations could be drawn from the observation of risk declining over calendar periods [15] or persisting over decades [29, 52]: interpretation of data should comprise possible beneficial effect of ongoing dietary treatment, not missing to consider the characteristic of the population over time. Actually, recognition of celiac disease in less severe cases or even in the absence of symptoms could have modified the overall clinical profile of the population and related prognosis.

When the degree of villous atrophy was evaluated with respect to the risk of lymphoproliferative evolution, no correlation was found. [52] Moreover, an increased risk of NHL and mortality was described in patients with non atrophic SB inflammation: the observation is, however, of limited interpretative value, given the lack of characterization of such histological damage, with risk declining over 5 years (effect of introduced GFD? Acute transient inflammation?).

A contributory effect of genetic predisposition is emphasized by the higher prevalence of HLA-DQ2 omozigosity in patients with CD complicated by type II RCD or EATL. [54] Genotyping could then be part of the strategy to stratify patients according to their potential risk.

Overall, evidence for a role of gluten avoidance in preventing complications is indirect and extrapolated from follow up observations of variable time duration, variable size of the studied population and with level of adherence to prescribed diet difficult to infer. [24, 47, 55]

1.3 Non responsive and refractory celiac disease

In the past, up to one fifth of CD patients were considered to be nonresponsive to GFD. Lack of agreement on the definition of non-responsiveness was obviously one of the causes for such a high

percentage. Actually 7 to 30% of celiac patients refer persistence of symptoms on GFD, requiring a deep evaluation of the clinical presentation. Firstly, accuracy of CD diagnosis should be reassess, including histological specimen examination and HLA typing in questionable cases. Secondly, adherence to GFD should be investigated, since gluten assumption is the most common determinant for symptoms persistence and consultation of a dietician should be considered. Approximately up to 5% of adult onset CD patients may have refractory celiac disease (RCD), defined as persistent symptoms and villous atrophy, despite scrupulous adherence to a gluten-free diet. The symptoms usually include diarrhea, weight loss, recurrence of malabsorption. Diagnosis of this condition is made after exclusion of concomitant extra-intestinal diseases (microscopic colitis, irritable bowel syndrome, pancreatic insufficiency), other small bowel disorders (lactose intolerance, bacterial overgrowth, inflammatory bowel disease, collagenous sprue, autoimmune enteropathy), including small bowel malignancies.

Refractory celiac disease may be classified as type I, in which there is a normal intraepithelial lymphocyte phenotype, or type II, in which there is a clonal expansion of an abnormal intraepithelial lymphocyte population. This aberrant population of intraepithelial lymphocytes is characterized by lack of surface expression of CD3/T-cell receptor (TCR) complexes and of CD8 but containing intracellular CD3ε and a clonal rearrangement of the gamma chain of T-cell receptor (TCRγ).

Practically, diagnosis of RCD has been proposed in case of symptoms relapse or continuation (chronic diarrhea and/or severe abdominal pain) with persistent malabsorption syndrome (decreased serum levels of iron, folate, vitamin B12 and/or calcium) in combination with histology showing persistent villous atrophy and increased numbers of IELs after 1 year of strict adherence to GFD, unless earlier intervention is required. RCD patients are almost always adults of 50 years and over. RCD II was defined by the presence of more than 25% $CD103^+$ or $CD45^+$ IELs lacking surface CD3/TCR complexes on flow citometry or >50% IELs expressing intracellular CD3ε but not CD8

in formol-fixed sections and/or the presence of detectable clonal TCR rearrangement in duodenal biopsy specimen.[21] RCD type II is associated with poor survival and between 45 and 60% of patients die within 5 years, despite therapies. Transformation to overt EATL occurs in 50-60% of cases within 5 years.

Ulcerative jejunoileitis was defined as more than three non-lymphomatous small intestinal ulcers, more than 0.5 cm sized, detected beyond the distal duodenum at push enteroscopy. [56] Ulcerative jejunoileitis may complicate and may precede the occurrence of overt T cell lymphoma in the presence of clonal IELs residents in intestinal ulcers.

T-cell lymphoma complicating CD is considered to derive from the clonal intestinal IEL population, given the expression of the β7 integrin HML1 (CD103) in lymphomnatous T cells, as in normal IELs. [57] Moreover, sequence identity of TCRγ between overt lymphoma tissue and intestinal biopsies have been reported and supports the hypothesis. [58]

From a therapeutic point of view, presently steroid therapy allows clinical improvement in the majority of patients; however, histological recovery occurs in 30-40% of subjects, without any impact on malignant transformation and with the onset of systemic complications drug-related. [59] When facing with steroid-resistance or steroid-dependence, Azathioprine, Ciclosporine and anti-TNF have been used without benefit, due the lack of effect on the clonal lymphocyte population and, on the contrary, with possible increase of the risk of lymphoma development (as in the case of combined Azathioprine anti-CD52 therapy). Till now, scanty and conflicting data are available on anti-CD52 monoclonal antibody (alemtuzumab) utilization in RCD II. Accurate identification of RCD II patients is needed in order to catch the chance of preventing EATL through early chemotherapy; cladribrine is a synthetic purine nucleoside showing good tolerance and moderate histologic and hematologic efficacy. [60, 61] High doses chemotherapies and autologous stem cells transplantation have been proposed in non responsive patients.[62, 63]

1.4 Markers of prognosis

1.4.1 Celiac disease

Recognition and prompt treatment of RCD is of utmost importance to ameliorate prognosis. Prediction of a complicated course would be necessary but not feasible, depending on the absence of markers of evolution. Thus, the importance of GFD response indicators should be stressed, in order to stratify patients and tailor targeted follow up programs.

Repetition of serological tests on GFD treatment is a way of monitoring adherence and response to gluten avoidance. However, correlation between antibodies disappearance and mucosal intestinal recovery is poor. Despite contradictory data, positive serology is assumed to reflect some gluten intake and low TTG levels do not predict histological remission. [64]

Specificity of persistent intestinal atrophy while on GFD is low and histological follow up of CD patients a prognostic indicator with poor accuracy. Actually, up to 70% of adult CD patients have been reported to maintain duodenal atrophy, [65] while a very strict minority of them will develop a complicated course. Guidelines recommend follow up duodenal biopsies in case of clinical non responsiveness, but routine upper endoscopy is not advised.

1.4.2 Refractory celiac disease

The characteristics of the intraepithelial lymphocyte population have been evaluated as far as prognostic implications are concerned. The identification of monoclonal TCRγ rearrangement has been associated with an increased mortality risk. [66] Oligoclonal rearrangements, instead, were reported in patients showing a benign course, not differently from the subjects with polyclonal TCR gene rearrangements. The study from Rubio-Tapia et al. [67] highlighted that cumulative risk factors, such as monoclonality, older age, total villous atrophy and low hemoglobin and albumin levels predicted 5-year survival rates in RCD patients. In the work by Arguelles-Grande et al., [66]

evaluating 73 RCD subjects, again gender, age, degree of villous atrophy, duration of CD, presence of non-EATL lymphoma, clinical presentation at diagnosis did not anticipate clinical deterioration. After adjusting for age, gender and serology, detection of <50% $CD3^+$ $CD8^+$ IELs by immunohistochemistry was a strong predictor of clinical course, with 5-fold higher risk of worsening within 2 years.

Most patients have negative CD serology at RCD diagnosis. However, persistence of CD related antibodies on GFD was observed in up to 30% of RCD patients. [21] Positive serology despite good compliance could be due to antibodies kinetics, presence of severe inflammation and destructive lesions or presence of coexisting autoimmune disorders leading to false positive CD serology. Patients with positive serology have been reported to have a shorter time to clinical worsening in the comparison with patients who had negative serology. [66] However, patients with <50% $CD3^+$ $CD8^+$ IELs and negative serology were at increased risk for anticipated clinical deterioration compared to patients with <50% $CD3^+$ $CD8^+$ IELs and positive serology who did not show an increased risk. In case of positive serology or polyclonal rearrangement with <50% $CD3^+$ $CD8^+$ IELs, a bad clinical outcome could be due to gluten exposure and an increase of $CD3^+$ $CD8^-$ reflecting an expansion of γδ IELs. On the other side, aberrant αβ IELs could account for the $CD8^-$ IEL expansion of seronegative patients. Flow citometry could be of benefit in elucidating the charateristics of the phenotype of the IELs subgroups.

Low haemoglobin levels and hypoalbubinemia may indicate a poor prognosis in RCD. [32, 67] Chronic elevated levels of transaminases were detected in about half of RCD patients in the series from Malamut et al.: in most cases, etiology remains unknown. [21]

Coexisting diseases were found in the majority of RCD patients: autoimmune diseases were not showed to be associated with a higher risk of clinical worsening; when CD-related and non CD-related diseases were looked at, infections were linked to a worse clinical outcome, while osteoporosis and microscopic colitis were reported in association with a lower risk of clinical

deterioration. Particularly, lymphocytic colitis was described in approximately one third of RCD patients (both type I and II). Lymphocitic gastritis was reported more frequently in type II than in type I RCD.

2. RISK OF SMALL BOWEL LYMPHOMA IN NOT RECOGNIZED CELIAC DISEASE: DATA FROM A REGISTERED POPULATION ACCORDING TO DIFFERENT COELIAC DISEASE PREVALENCE.

2.1 Introduction

Up to now the gluten-free diet (GFD) is the unique available therapy for coeliac patients and it is suggested to eliminate symptoms, normalize intestinal mucosa and, chiefly, prevent CD complications.[24, 53]

The most fearful complications of CD are malignancies of the gastrointestinal (GI) tract, mainly non-Hodgkin and enteropathy associated T-cell lymphomas (NHL and EATL). Risk to develop GI lymphomas compared to the general population is different in the published studies. Moreover, some studies suggest that GFD and early diagnosis prevent the theoretical evolution of the chronic intestinal inflammation to the malignancy of the immunological cells by the elimination of the environmental stimulating antigen (gluten). [25, 53] Considering that CD is a common (1:100) and underdiagnosed disorder (1 out of 10), [68-71] the prevention of its harmful complications is a relevant topic with high social impact, involving a policy of early diagnosis and strict treatment compliance which imply social costs and personal sacrifice.

The aim of this study is to estimate the risk of GI B- and T-cell lymphoma in undiagnosed CD in a province of the Northern Italy.

2.2 Patients and methods

2.2.1 Collection of data

Data were collected from 1st January 1999 to 31st December 2004. The Varese Province was chosen because of the stability of its population and availability of cancer registry.

2.2.2 Cancer registry

The Lombardy Cancer Registry is a population-based registry, covering all residents in the province of Varese, Northern Italy (mean 815,362 between 1999-2004). Collected data are tumor site and histological type according to ICDO3 classification (International Classification of Disease for Oncology, WHO, 2000), diagnosis date, hospital where the diagnosis was made and demographic information. The Registry is part of the network of the International Agency for Research on Cancer (IARC) from the seventies: its data were used for large international collaborative studies (Eurocare, Epic, Eurochip, Europreval). The registry uses all electronic source files (hospital discharge files, death certificates, regional health files, and pathology reports) and a proven-case generation methodology.[72-74]

The completeness and diagnostic accuracy of the registry for the lymphoproliferative tumors is 98,7%. [74]

All gastric mucosa associated lymphoid tissue lymphoma (MALT) were excluded from the analysis.

2.2.3 Selection of coeliac cases

The cohort of patients with CD (ICD9-CM, International Classification of Disease, WHO 1997 – code 579.0) was obtained by identifying, through computerized search of the four Pathology Departments of the Province of Varese, histological reports from 1st January 1999 to 31th December 2004. Only cases with a duodenal pathological report compatible with a grade 3 of the Marsh classification [75] were included.

The CD prevalence is assumed stable in the period under study and in the 20 years before to take into account the latency between exposition (CD) and cancer.

2.2.4 Estimation of Lymphoma risk

The expected cases of intestinal lymphomas in the coeliac population were estimated multiplying the risk of lymphoma in the general population for the number of coeliac prevalent cases, accordingly to different CD prevalence (1:50, 1:100, and 1:200) and adjusted for the reported relative risks of lymphoma in the coeliac patients. We assumed the simplifying conditions that the prevalence of CD in the Varese population was constant and the fraction of risk of lymphoma due to undiagnosed CD in the general population was that reported in the literature. [25, 29, 76-78] The risk of lymphoma was computed diving the number of lymphoma cases in the study period by the population under risk, that is to say the coeliac patients. Because the fraction of lymphomas attributed to CD is not precisely known, we estimated as all the GI B- and T-cell lymphomas were attributed to the CD and then other estimates with different assumption.

Cases with coincident diagnosis of lymphoma and CD have been excluded from analyses.

2.3 Results

The population of Varese province had a 3.7% increase in the period of time considered; in 1999 it was composed by 806,323 inhabitants and in 2004 by 836,440 (mean number of inhabitants per year 815,362, 51·7% females, mean age 43·2).

From 1999 to 2004 1,180 NHLs have been observed with a mean number per year of 197 (Table1); 69.0% were extraintestinal B-cell NHLs, 19.0% GI B-cell NHLs, 11.0% extraintestinal T-cell NHLs and 1.0% GI T-cell NHL. Males represented the 54.5% (n=643) of patients with NHL, the 50.6% of GI NHL, the 50.4 of GI B-cell NHL and the 55.5% of the GI T-cell NHL (no statistical difference between males and females). Twenty-nine gastric MALT in 1999, 10 in 2000, 11 in 2001, 15 in 2002, 11 in 2003 and 15 in 2004, were excluded from our analysis.

The incidence (100,000 persons/year) of NHLs and subtypes was stable in the 6 years analyzed, being 24.1 for NHL, 4.8 for GI NHL, 21.4 for B-cell NHL, 4.7 for GI B-cell NHL, 2.7 for T-cell lymphomas and 0.2 for GI T-cell lymphoma.

In the 6-years considered, according to the data from the Pathology Departments, a total of 6618 subjects (M/F 2947/3671, mean age males 52.6 ± 20.0, mean age females 49.3± 21.0, total mean age 50.8± 20.1) underwent duodenal endoscopic sampling, and 326 (4.9%) resulted compatible with CD (mean yearly diagnosis 54, M/F 78/248, mean age males 30.6 ± 18.6, mean age females 31.8 ± 17.7, total mean age 31.5± 17.9). The prevalence of diagnosed CD in the Varese population was 0.04%. In coeliac group a single case of GI B-cell NHL was observed; however, the patient, a 59 years old female, had been excluded from the data analysis because diagnosis of CD and Ki-1 positive anaplastic GI NHL were coincident.

According to the real incidences of GI lymphomas observed in the investigated population, we applied different settings of CD prevalence (1:50, 1:100 and 1:200), as it should be from the studies investigating the presence of CD in the Caucasian populations, usually reported from 1:200 to 1:50. [69, 79-81]

The first analysis is reported in Tables 2 and 3; in these tables are showed the expected cases of GI B- and T-cell NHLs in the coeliac cohort, according to the theoretical prevalence of CD in Varese Province, given different relative risks (RRs) derived from literature. Moreover, in the tables the impact (percentage) on the total number of observed GI NHLs is reported. In Tables 4 and 5 the risks to develop GI B- and T-cell NHLs in the CD theoretical population, calculated for different prevalence, are reported. The risks are defined for different percentages of CD associated GI B- and T-cell NHLs; thus, in every case of CD prevalence setting and percentage of NHLs directly induced by CD, the corresponding risk is showed.

2.4 Discussion

The present study shows that undiagnosed/untreated coeliac patients have the same risk of developing GI lymphomas than the general population and thus this risk is not associated with gluten exposure.

The rate of lymphomas registered in the Varese population is in accordance with the prevalence and incidence reported in other population studies. [82]

Also the prevalence of diagnosed CD is in line with those described by other authors and, in particular, it is confirmed that only a few number (1:10) of coeliac patients receive a correct diagnosis. [83]

Consequently, the largest part of the coeliac patients remain undiagnosed and untreated, continuing a regular dietary consumption of gluten, exposing them to a theoretical increased risk of developing lymphoproliferative complications [25]; in fact, the most qualified hypothesis suggests that the longstanding gluten exposure is the factor responsible for the development refractory CD, EATL and in general NHL. [58, 84]

Different studies attempted to estimate the risk of lymphoma in diagnosed coeliac patients but results were not conclusive with a range of risks varying from no risk [55, 76] to 300-fold increase. [4]

In our opinion, the main bias of the studies presenting extremely high risks to develop lymphoproliferative disorders in CD is the selection of the CD patients frequently derived from selected cohorts referring to tertiary centres and thus, not reflecting a general population "scenario". To support this hypothesis, it is notable that some these cohorts often report also the association with other rare tumours as the oesophageal one suggesting an apical selection of the patients. [4]. Other studies enrolled cohorts deriving from hospitalized patients diagnosed in the seventies when CD was considered extremely rare and thus probably only heavily symptomatic patients with malabsorption received a correct diagnosis.[24]

Moreover, CD and lymphoma are often diagnosed at the same time or within 3 years and a reverse causality could not be excluded and some other studies were conducted on small number of selected patients, or serologically diagnosed. [25] In fact, from most recent studies the RR is attested from 1 to 10 in diagnosed CD and it seems especially attributable to patients with a late CD diagnosis after the 40 year of age. [9]

From our study in Varese province, we can say that if all the observed cases of GI B- and T-cell NHLs were attributable to CD, the RR should be 200, 100 or 50 in populations with theoretic CD prevalence of 1:200, 1:100 or 1:50, respectively. However, we know that the proposed hypothesis "100% of NHL are associated to CD" is a paradox, very far from reality.

Several studies, although with different methodologies and conflicting results, estimated the prevalence of CD in cohorts of patients affected by intestinal lymphomas. Majority of these studies identified patients with an already known diagnosis of CD and patients with a diagnosis of CD occurred when screened at the time of the lymphoma; taking into account the problems regarding the coincidental diagnosis, this latter group could be assumed as the prevalence of undiagnosed CD in the GI NHL population. The prevalence of hidden and undiagnosed CD in GI NHLs resulted 2.0% in the study by Catassi et al. [25], 1.1% in a European multi centre study [77], 0% in studies conducted in Spain and Turkey [76, 78]; thus, it reasonable to assume a prevalence of undiagnosed CD among GI NHL ranging from 0% to 2%, probably close to 1%. [25, 29] Consequently, assuming that 1–2% of GI NHL is CD-associated, the RR of GI B- and T-cell NHL for coeliac patients ranges from 1.0 to 2.0 for a CD prevalence of 1:100 (Tables 2 and 3). Thus, the risk-year for 10,000 coeliac patients (prevalence 1:100) in Varese Province to develop GI B- and T-cell NHL varies from 0.46 to 0.93 and from 0.02 to 0.04, respectively.

Our data demonstrate that the risk to develop a GI NHL in a theoretical population of undiagnosed and untreated CD patients is low, not higher than that observed in diagnosed and treated CD and lower than that present in some studies from the literature. [4] They are in line with the recent

results from Ludvigsson et al. [9] who show a 3- and 2-fold increased Hazard Ratio (HR) of lymphoproliferative malignancies in CD and duodenal inflammation respectively, and no risk for patients affected by potential CD (that is positive CD antibodies without duodenal histological damage). They suggest that the possible risk factor could be the inflammation itself and not CD. Although obtained with a different approach, our data are also in line with those presented by Lohi et al. [85] They evaluated the malignancy risk of undiagnosed CD by searching for CD specific autoantibodies in stored sera from a general population; in serologically positive patients they did not find an excesses of lymphomas or mortality and a 1% prevalence of CD was confirmed.

The absence of a serologically screening of our population could be considered a point of weakness to investigate the hidden part of the CD iceberg; however, also the serological presence of CD autoantibodies could lead to some mistakes as showed in the study by Mearin et al. [77] where the 50% of NHL patients with positive anti endomysium antibodies did not present a villous atrophy suggesting an higher rate of false positive in patients with haematological malignancies.

For clinical purpose, to better define the meaning of the RRs showed above, if we consider a hypothetical Varese population composed by 99,000 healthy subjects (risk for GI NHL 4.8 per 100.000 person/year) and 1000 undiagnosed and untreated CD patients (prevalence 1%) (risk for GI NHL 6.2 per 100,000 person/year) the non-CD subjects generates 4.7 GI NHL per year and the undiagnosed CD subjects generates 0.062 GI NHL; thus, all the CD population will take 16 years to develop only one GI NHL.

Moreover, the present study induces some reflections on the role of gluten on the development of NHL in patients affected by CD. In fact, data about gluten exposure, measured as years of exposition or in terms of compliance to the GFD, are conflicting and scarcely precise because of lack of objective evaluation of completeness of the diet. [25, 53] Even if the widely accepted and logically intriguing idea that the gluten-induced chronic stimulation of immunological cells in the

intestinal mucosa of undiagnosed CD is at the basis of the development of a refractory state and NHL, looking at the evidence, more data are necessary to support this hypothesis.

From a practical point of view, our study does not support the need to screen general population and asymptomatic subjects in order to prevent possible lymphomas and further data on the natural history of CD could be useful to program future national and familiar screening.

3. A CAPSULE ENDOSCOPY AND DOUBLE BALLOON ENTEROSCOPY SEQUENTIAL APPROACH FOR EARLY DETECTION OF GASTROINTESTINAL TUMORS IN CELIAC DISEASE: A PROSPECTIVE TRIAL

3.1 Introduction

The epidemiological characteristics of SB malignant complications and the knowledge of the supposed associated risk factors (age at CD diagnosis, mucosal healing, compliance to GFD) have led to the following considerations: (i) the risk of gastrointestinal malignancy in CD is not homogenously distributed; (ii) attention should be especially paid to those CD patients with demographic and clinical features that identify them at presumably higher risk of having a neoplastic complication; (iii) for such a subset of CD patients, an early diagnostic strategy of SB tumors is yet to be evaluated.

The difficulty in SB exploration used to be the major problem for the early diagnosis of intestinal tumors. The introduction of SB capsule endoscopy (SBCE) and device-assisted enteroscopy (DAE) facilitated the study of the SB mucosa.

Small bowel capsule endoscopy is a safe investigation allowing the direct visualization of the SB with a low invasive technology. Standard indications to perform SBCE are the investigation of obscure gastrointestinal bleeding, the assessment of SB lesions in known or suspected Crohn's disease, screening and surveillance in familial polyposis syndromes, assessment and localization of SB tumours; moreover, the enteroscopic techniques have been applied to both the diagnosis and management of patients with complicated CD, even if the data currently available are mainly obtained from retrospective investigations.

Device-assisted enteroscopy is a system allowing the insertion of dedicated endoscopes through the SB. DAE is mainly represented by double-balloon enteroscopy (DBE): the system consists of a balloon assembled on the distal end of the enteroscope and a balloon on the overtube; its use

requires a series of steps employing a push and pull technique. Indications are almost the same of SBCE, with the additional chance of performing biopsies and treatment of a previously diagnosed disease (polipectomy, dilatation).

Based on the aforementioned considerations, the present study aimed at prospectively evaluating the application of selection criteria (including the presumed clinical risk factors for SB malignancies in CD) and the diagnostic yield of SBCE and DBE, in the early detection of intestinal malignancies for this CD cohort.

3.2 Methods

3.2.1 Patients

From June 10, 2011 to November 30, 2014, all consecutive CD patients attending the Center for the Prevention and Diagnosis of Celiac Disease – Gastroenterology and Endoscopy Unit at the Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico (Milan, Italy) were prospectively evaluated. According to the international guidelines, [5] CD diagnosis was based on the histological evidence of duodenal atrophy (a type 3 lesion according to the Marsh-Oberhuber classification).[6] The presentation type was categorized as classic (diarrhea, weight loss, longitudinal growth retardation), mono/paucisymptomatic (dyspepsia, anemia, hypertransaminasemia, osteopenia) or associated with the presence of dermatitis herpetiformis (DH).

The patients were enrolled according to the following inclusion criteria:

(i) presence of alarm symptoms/signs, either at diagnosis or during the follow-up;

(ii) lack of compliance to GFD (defined as the conscious and regular gluten ingestion for at least 2 years);

(iii) positive findings at radiological SB study.

The study was approved by the local ethical and was carried out in accordance with the Declaration of Helsinky. Patients fulfilling the inclusion criteria gave their written informed consent to be

enrolled in the study and then underwent the following work-up: routine blood tests (i.e. full blood count, ferritin, transaminases, alkaline phosphatase, gamma-GT, glycemia, cholesterol, triglycerides, TSH); anti-endomisium and anti-transglutaminase antibodies; endoscopy of the upper GI tract with at least four oriented duodenal biopsies; determination of histological grading (Marsh-Oberhuber's classification) and CD3/CD8 immunoistochemistry; SBCE; DBE in case of positive findings at SBCE or when SBCE was contraindicated. Further investigations were carried out when clinically indicated. In details: TCR γ rearrangement analysis in case of suspected RCD or intestinal lymphoma; HLA typing to determine a genetic CD susceptibility; colonoscopy with multiple biopsies when suspecting colonic tumors, inflammatory bowel disease, or microscopic colitis; ultrasonography as a first step evaluation in case of abdominal symptoms. Furthermore, CT or MR enterography were performed for tumor staging or in case of intestinal obstruction.

SBCE (Pillcam SB3, Given Imaging, Yoqneam, Israel) was performed after intestinal cleaning with a 3-litre polyethylene-glycol (PEG)-based solution (to be assumed the day before the examination) and overnight fasting. Patients with a cardiac pacemaker underwent examination under continuous cardiac monitoring. The Given Imaging recording system was positioned according to the manufacturer's instructions; data were downloaded on a dedicated computer workstation and analyzed by a dedicated software (Given Imaging, Yoqneam, Israel). In those patients who had undergone major abdominal surgery or presented symptoms consistent with possible intestinal obstruction, SBCE was preceded by the evaluation of the intestinal canalization by patency capsule (Agile, Given Imaging, Yoqneam, Israel). As from Given Imaging specifications, the examination time with Pillcam SB3 was at least 9 hours. All registrations were conducted till battery exhaustion. The same physicians performed SBCE and DBE, and were in charge of CD management. In case of positive SBCE findings, DBE (Fujinon, Saitama, Japan) was then carried out. The following endoscopic features were considered for histological evaluation: mass lesions, ulcers, nodules or nodular mucosal patterns, extensive (beyond 30% of SB transit time) or patchy distribution of

atrophic features (mosaicism, scalloping, fissures). The route for insertion was planned according to the estimated site of the lesion as from SBCE (cutoff at 70% of capsule progression). Histological and immunohistochemical evaluations (CD3, CD8) were performed on the samples obtained from both the lesion and the adjacent mucosa.

One-hundred and sixty-five non-celiac patients, matched for gender and age, and undergoing SBCE for obscure GI bleeding (overt or occult), were included as a control group for the SBCE diagnostic yield evaluation. The incidence of the tumor types diagnosed in the CD cohort was compared to that of a registered population (from the Lombardy Regional Cancer Database) of the Varese province (815,362 inhabitants), assumed as an index of incidence in the general population. Subject with CD were excluded from the above mentioned database. The tumor sites and histological types according to the ICDO3 classification (International Classification of Disease for Oncology, WHO, 2000) are reported in the database with a level of completeness and diagnostic accuracy of 98.7%. [74]

3.2.2 Statistical analysis

Statistical analyses were performed using SPSS ver.18. A P value of <0.05 was considered statistically significant (significance level for the tests: 5%, two tails). The sample size was calculated assuming a 5% prevalence of malignancies in the CD cohort. The normal distribution of the sample was verified through the Kolmogorov-Smirnov test. Continuous variables were analyzed with the ANOVA Oneway variance test or with the non-parametric Kruskal-Wallis test. The significance level was further verified by multiple comparison analysis (Turkey or Mann-Whitney's test). Categorical variables were compared with the X^2 or Fisher's exact test.

3.3. Results

3.3.1. Patients profile

One hundred and five consecutive patients were enrolled (20 males); mean age at diagnosis was 39.3±18.2 years, mean age at enrollment 46.8±14.0. When first diagnosed, 73 patients (69%) showed a classic clinical presentation, 28 (27%) were mono/paucisymptomatic and 4 (4%) had DH. Twelve cases (11%) tested negative at serology for CD: three had a selective IgA deficiency, and all presented DQ2 and/or DQ8 haplotypes. The clinical and demographic characteristics of the enrolled subjects are detailed in Table 6.

Overall, the present series involved (i) seven patients (7%) with positive findings at trans-sectional imaging examination, (ii) 16 patients (15%) poorly compliant to GFD, and (iii) 82 (78%) having alarm symptoms or signs; this latter group included 29 patients with iron deficiency anemia, 6 with B symptoms consistent with hematologic disorders, and 47 with severe gastrointestinal symptoms.

Eighty-six subjects (82%) entered the study after at least one year on GFD: among them 51 (49%) showed duodenal atrophy (Marsh-Oberhuber grade 3) at the follow-up histological evaluation. Six patients had type I RCD and six were diagnosed with type II RCD.

3.3.2. Endoscopic findings

One hundred and sixteen capsule endoscopy investigations were performed (11 patients underwent examination twice, due to follow up indication or incomplete small bowel exploration in nine and two cases, respectively). Capsule swallowing was uneventful in 115 cases (99%), whereas in a single one the capsule was endoscopically positioned in the duodenum since the first attempt failed due to a bulky gastric bezoar. One patient did not perform SBCE but directly underwent DBE due to a suspected intestinal obstruction. Capsule evaluation was completed in 99 cases (94%). Registration was stopped in the stomach in one patient with a gastric source of bleeding. The mean SB transit time was 266±93 min and it resulted slower in the RCD group (333±102min, P=0.01). At

SBCE mucosal abnormalities were detected in 71 capsule examinations (68%): in detail, a jejunal stenosis (adenocarcinoma), three ileal submucosal bulges (neuroendocrine tumor in one case), a gastric bleeding mass (adenocarcinoma), granular gastric mucosa (a MALT lymphoma), SB mucosal nodular alterations (n=2), SB small erosions (n=15) or ulcers (n=4) and intestinal mucosal atrophy (n=44). SBCE retention was observed in the patient with jejunal stenosis and capsule were retrieved during subsequent surgical intervention.

Overall, 16 patients underwent DBE due to positive findings at SBCE (following the evidence of extensive or patchy distribution of mucosal atrophy in seven cases, severe ileal nodularity in two, ileal ulcers in two patients, submucosal bulging in three cases) or when SBCE was contraindicated for suspected obstruction (one case). DBE and histological evaluation led to the diagnosis of a neuroendocrine tumor and a jejunal adenocarcinoma in the latter two patients (tumor diagnosis in 12% of total DBE examinations). In a single case a mild-post DBE pancreatitis was observed.

Colonoscopy was performed in 71 patients (68%), with detection of small non-dysplastic polyps in four cases and microscopic colitis in other five cases (5%).

3.3.3 Comparison with control groups and risk factors

In this group of celiac patients, the diagnostic yield of SBCE and DBE for malignancies was 2.9%. When the SB was investigated with SBCE for obscure gastrointestinal bleeding in non-celiac patients, tumors were detected in 0.6% (P=NS).

When considering the registered population of the Varese province, the incidence rate of SB malignancies (adenocarcinoma and neuroendocrine tumors) was 2/100,000 inhabitants per year. As from the results of the present study, the calculated incidence rate of the same SB tumors in CD selected patients was 836/100,000 inhabitants per year. Accordingly, when compared to the registered population, the relative risk for SB malignancies in the selected CD cohort was 410 (CI 95% 102-1640, P<0.0001).

The diagnosis of SB malignancy was not associated with gender or GFD compliance (P=1, Fisher's exact test). Similarly, no association was found between a malignancy diagnosis and the presence of duodenal atrophy (P=1). Evidence of SB tumors was more frequent in the first year after diagnosis (P=0.0001). A trend toward an association between SB malignancies and the presence of anemia was observed (P=0.09, Fisher's exact test; hemoglobin levels in malignant cases vs. patients with negative neoplastic findings, P=0.05).

3.3.4 Patients follow-up

Enrolled patients underwent clinical follow-up (twice a year) for a mean period of 20±13 months after SB evaluation, according to their entry date. Patients with negative neoplastic findings at enteroscopy did not present new symptoms or SB complications at follow-up. The described cases of SB malignancies were negative for disease recurrence after 29, 41, and 19 months, respectively. Exitus was reported at follow up in a 69 years old patient due to acute cardiac failure; one patient died 5 years after the diagnosis of type II RCD due to secondary multi-organ failure.

3.4 Discussion

The present study was aimed at evaluating the selection criteria associated with increased risk of SB malignancies in CD patients and the use of SBCE and DBE for the detection of such malignancies. In that context, SBCE and DBE allowed to diagnose three SB cancers: two jejunal adenocarcinoma and an ileal neuroendocrine tumor. As tumor incidence is not homogeneous in the CD population and the diagnosis of this complication is often late and associated with a poor prognosis, the identification of the subset of CD patients deserving an early diagnostic approach is mandatory and even more urgent based on the availability of new high-performing tools for SB examination.

Small bowel capsule endoscopy has been considered of relevance in the evaluation of CD patients [18,19]. Available data on the SBCE use in a CD population are negatively influenced by the

retrospective design of the majority of the studies. Presently, the surveillance of RCD patients represents the main indication. Capsule endoscopy has proved to be useful in detecting enteropathy-associated T-cell lymphoma (EATL) and ulcerative jejunoileitis among RCD patients [20-22]. When the SBCE examinations in treated symptomatic CD patients were reviewed, diagnostic yield for ulcerative jejunoileitis and SB malignancies (EATL, adenocarcinoma) was 4.8% and 6.25% in the series of 42 and 48 patients, respectively [21,23,24]. In a further study by Maiden [25] nineteen CD cases were analyzed showing in two patients multiple ulcers consistent with ulcerative jejunoileitis, although no histological data were reported. Small isolated ulcers were detected in 3 out of 50 CD patients in the study by Collin et al. [21] but, similarly, no histological data are available. Ulcerative jejunoileitis is arbitrarly defined to date as the finding of at least three jejunal ulcerations at enteroscopy. [26] The suspicion of lymphomatous complications is considered if strictures and/or multiple ulcers are observed. Little is known about the predictive value of minor mucosal lesions detected at SBCE. Device-assisted enteroscopy is the only technique that allows for the adequate sampling of the SB when facing with mucosal lesions detected at SBCE, but little is known about the SBCE-DBE sequential use in patients with somewhat complicated CD.

Small bowel capsule endoscopy has been evaluated by Culliford et al. [27] in CD patients showing the persistence of gastrointestinal symptoms, history of SB cancer, long standing CD with lack of dietary compliance and/or iron-deficiency anemia not responsive to oral supplementation. Forty-seven patients on gluten free diet were studied with evidence of ulcerations in 21 cases, nodularity in six and cancer, polyp, stricture, intussusception and submucosal mass in one case, respectively.

A prospective study has recently evaluated the use of SBCE in equivocal CD cases and 69 symptomatic CD patients undertaking gluten free diet: in the latter cohort SBCE was positive in eight patients (mass lesion, extensive atrophy, ulcers); push or DBE enteroscopy confirmed the diagnosis of two EATL, four type I RCD, one ulcerative jejunoileitis; the polypoid mass at surgical removal was fibroepithelial [28].

In the present series three tumors were detected among the patients enrolled with iron deficiency anemia. Indeed, the results strengthen the relevance of unexplained iron deficiency anemia as an alarm sign in CD patients too, especially when severe and/or unresponsive to both gluten free diet and oral iron supplementation. In addition to a selective iron deficiency due to malabsorption, anemia could indicate a concomitant disease either at CD diagnosis or during an on-going gluten free regimen.

The association of neuroendocrine tumors and CD is doubtful. If the hyperplasia of entero-chromaffin cells has been demonstrated in CD [29] only case reports have been published describing CD subjects with neuroendocrine tumors. From this point of view, the finding of a neuroendocrine tumor in our series can be considered sporadic rather than a CD complication.

The presented cases of SB tumors detected by SBCE and DBE had a favorable prognosis, when compared to literature data, supporting the impact of early diagnosis.

Interestingly, SB malignancies were detected in patients diagnosed in adulthood (at 48, 31 and 38 years respectively), in line with the large Swedish population-based study [3]. No malignancies were detected among non-compliant patients, even if the limited size of this subgroup precludes any definite conclusions. The high frequency of tumor diagnosis in the first year after CD diagnosis may reflect ascertainment bias, but it also represents the need for close follow up of those patients without a prompt response after starting GFD.

Computerized tomography enterography and magnetic resonance enterography (a radiation-free technique that is advisable in young prevalently female cohorts) have showed an improved accuracy in visualizing the SB wall. On the other hand, the growing use in the last decade of SBCE and device-assisted enteroscopy has enabled the direct visualization and histological study of SB mucosa. As a consequence, while a suitable accuracy for SB tumors has been described for the radiologic techniques, enteroscopy can better detect small lesions, confined to the mucosal layer

[30-32]. Since the expected SB complications in CD (namely ulcerative jejunoileitis, lymphoma and adenocarcinoma) share a mucosal origin, endoscopy appears the most appropriate approach when looking for any early stage SB malignancies.

Small bowel capsule endoscopy was followed in two cases by the diagnosis of a gastric adenocarcinoma and a gastric MALToma, respectively. This is in line with the known possible occurrence at SBCE of findings related to the upper or lower gastrointestinal tract, missed by conventional endoscopy.

Double-balloon enteroscopy was performed in 16 patients following SBCE, with the diagnosis of a neuroendocrine tumor despite negative findings at computerized tomography enterography. It is worth noting that to date a standardized classification of the endoscopic appearance of the early-stage neoplastic SB mucosal lesions is missing. In case of nodular mucosal alterations, extensive and/or distal patchy atrophy, ulcerative lesions at SBCE in patients with clinically suspected RCD or intestinal lymphoma, DBE was planned and the histological excluded an underlying neoplasia. Thus, the poor predictive value of SBCE represents a major limit, suggesting that better understanding of the endoscopic appearance of early stage malignant lesions is needed. One patient with contra-indications to SBCE underwent DBE with evidence of jejunal adenocarcinoma.

The enteroscopic techniques applied to our series showed a high diagnostic yield (even if not statistically significant in the comparison with non-CD patients undergoing SBCE for obscure gastrointestinal bleeding), presumably confirming an increased baseline risk in the selected cohort.

When the incidence rate of SB malignancies was compared to that observed in the registered population of the Varese province, the calculated relative risk for intestinal malignancy in the selected CD patients was 410 (CI 95% 102-1640, $P<0.0001$).

However, when looking at these data a referral bias should be taken into consideration, as our patients were enrolled among those attending a tertiary center, i.e. in a cohort already selected rather

than in an unselected CD population. Another possible bias would be the presence in the Varese population of hidden CD cases, but, as previously demonstrated [9] this would not be expected to influence the results.

As far as diagnostic accuracy is concerned, the high diagnostic yield of SBCE and DBE for tumors is worth noting together with the high number of patients undergoing DBE in the present series. Furthermore, close follow-up has not evidenced other tumors previously undetected.

The present study represents a prospective attempt at the early detection of SB malignancy in complicated CD. Further research on the identification of the clinical and endoscopic characteristics of the “at risk profile” is needed.

4. DOUBLE-BALLOON ENTEROSCOPY IN CELIAC DISEASE: EXPLORING INDICATIONS AND DIAGNOSTIC GAIN IN THE EXPERIENCE OF TWO EUROPEAN TERTIARY REFERRAL CENTERS

4.1 Introduction

Enteroscopic techniques as device assisted enteroscopy (DAE), usually preceded by small bowel capsule endoscopy (SBCE), are a valuable tool for the study of the small bowel (SB) and for the diagnosis and treatment of SB diseases. Mucosal lesions described and localized at SBCE can be then histologically characterized or treated by DAE. The described stepwise approach has been recognized as cost-effective in the management of patients undergoing SB evaluation for obscure gastrointestinal bleeding; at present, however, data in patients with celiac disease (CD) are only scattered.[86, 87]

The follow up of a known refractory state represents the most frequent indication to SB endoscopic investigation in CD population.[88],[89] Apart from this, the selection of celiac patients who deserve an accurate work-up for suspected SB malignant complications is still based on clinical experience, since evidence-based data are lacking.

Notwithstanding a growing experience with SBCE in complicated CD patients, the actual agreement in the definition of related mucosal alterations is still low[90] and the clinical relevance of subtle abnormalities is unknown, making histological sampling obtained with DAE mandatory.

According to the present knowledge, celiac enteropathy is characterized by aboral decreasing severity of the intestinal damage but less is known concerning the distribution of mucosal lesions during GFD.[91],[92] Noteworthy, EATL occurs more frequently distally in the small bowel[93] and also in the presence of non atrophic lesions in the adjacent mucosa.[94]

Again, ulcerative jejunoileitis (UJ) has been arbitrarily defined by the presence of at least three mucosal ulcerations at enteroscopy;[56] however, the poor specificity of the endoscopic finding

requires histological integration to make a correct diagnosis (EATL, Crohn's disease, infectious or drug related conditions).

In line with these assumptions, we should consider (a) a complete longitudinal exploration of the SB is necessary in complicated CD patients; (b) a non atrophic duodenal histology could underestimate the presence of SB complications; (c) SBCE represents a well tolerated diagnostic tool in this context but histological examination through DAE is often needed to understand the possible pathologic meaning of the detected findings.

All in all, the sequential use of SBCE and DAE is a potentially useful strategy in complicated CD, providing that proper indications to enteroscopy are acknowledged and applied.

Present series was aimed at evaluating, in CD patients investigated for suspected SB complications, the usefulness of DBE and the predictive value of the related SB endoscopic features.

4.2 Methods

4.2.1 Patients

Celiac patients undergoing DBE between 1st February 2009 and 31th May 2014 at the Center for the Prevention and Diagnosis of Celiac Disease at the Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico Milan, Italy and Gastroenterology and Liver Unit, Royal Hallamshire Hospital, Sheffield, UK were retrospectively evaluated. All patients have been diagnosed as celiacs according to the histological evidence of duodenal atrophy [5] and had undergone SB evaluation in order to exclude intestinal complications. Poor adherence to GFD was defined as regular and conscious ingestion of gluten for at least one year.

The study was approved by the Ethical Committee (approval n° 2271).

Prior to DBE, SBCE (Pillcam SB2, Given Imaging, Yoqneam, Israel) was performed when feasible. In cases of suspected bowel obstruction (previous major abdominal surgery, symptoms suggestive for altered intestinal canalization), SB patency was preliminary tested with Agile capsule (Given Imaging, Yoqneam, Israel). The test was considered negative for obstruction if the degradable capsule was retrieved undamaged and in the absence of gastrointestinal symptoms.

In cases of positive findings at SBCE or when capsule endoscopy was contraindicated, DBE (Fujinon, Saitama, Japan) was carried out. The route of insertion was established according to the localization of the lesion at SBCE (cut-off at 75% of capsule progression).[95] Double-balloon enteroscopy was performed under conscious sedation. In presence of significant comorbidity, an Anaesthetist was actually present. Mucosal sampling from the targeted lesion and the adjacent mucosa was performed for histological evaluation and TCR gamma rearrangement.

Radiological investigations (SB targeted ultrasound, computed tomography enterography, magnetic resonance enterography) were reported when carried out.

Demographic data of the studied cohort were compared with a database of 1000 non complicated celiac patients attending the Center for the Prevention and Diagnosis of Celiac Disease, Milan.

4.2.2 Statistical analysis

All the assumptions were verified using SPSS ver. 18 (IBM SPSS, La Jolla, CA), and a P value <0.05 was considered as statistically significant (significance level of the tests 5%, two tails). Continuous non parametric variables were analyzed with the Mann-Whitney's test. Categorical variables were compared by χ^2 or Fisher's exact test. Data were expresses as mean (± SD).

4.3 Results

4.3.1 Patients

Twenty-four CD patients (12 males, p=0.01 *vs* control database) underwent DBE. Both mean age (years) at CD diagnosis 37 (±20) *vs* 27 (±18) and at SB evaluation 47 (±15) *vs* 38 (±13) were significantly higher in the DBE group compared to controls (p<0.01). The body mass index (BMI) of the enrolled patients was 21 (±4) kg/cm^2 (Table 6).

Four patients were negative for celiac serology at diagnosis. HLA class II determination, anti-enterocytes antibodies serum immunofluorescence, fecal and blood tests to exclude infective etiologies, endoscopic and imaging studies in order to differentiate inflammatory and neoplastic causes of villous atrophy were performed in these patients. Sixteen patients were diagnosed due to classic symptoms and eight patients were paucisymptomatic. Overall, poor adherence to a GFD was registered in seven patients. Median duration of follow up after CD diagnosis was 1.75 years (range 0-70).

4.3.2 Endoscopic studies

Twenty-seven DBE were carried out. Indications to SB evaluation were (I) the follow up of a known RCD (n=9), (II) suspicion of SB complications due to (IIa) gastrointestinal symptoms (n=6), (IIb) severe iron deficiency anemia (n=6) and (IIc) long standing lack of dietary adherence (n=3). All DBE were performed after SBCE, according to the presence of mucosal alterations at SBCE, except for two cases (due to symptoms suggestive of bowel obstruction). Oral steroid therapy was ongoing in five RCD patients at the time of SB evaluation; four of these patients were concomitantly given Azathioprine.

Small bowel capsule studies were preceded by patency capsule evaluation (Agile) in seven cases: capsule retrieval was not possible in one patient who reported abdominal symptoms during the test. Magnetic resonance enterography and DBE from both approaches were performed in the patient

with negative results. Small bowel capsule exploration was incomplete in six patients (33%): one of these cases presented with a stricturing mass and other four patients had RCD (incomplete SB capsule evaluation in 44% of RCD cases; p=0.18, Fisher's exact test). Mean SBCE transit time was 281 (±109) minutes. No cases of retention were registered.

Double balloon enteroscopy was performed via the oral route in 16 cases, with a mean insertion depth of 223 (±111) cm. Eleven enteroscopies were performed from the anal approach (three patients underwent both approaches, with the achievement of an "end to end" exploration in one). In one case DBE was not feasible due to the failure of the cannulation of the ileo-caecal valve. The mean length of the SB explored via the anal route was 122 (±106) cm. Mean duration time of DBE for both antegrade and retrograde approach (with no difference between the two) was 68 (±20) minutes. A case of mild post-DBE pancreatitis was observed in one patient (female, 36 year old, BMI=18).

4.3.3 Small bowel findings

A diagnosis of jejunal adenocarcinoma was made in two patients, one from Sheffield and one from Milan. In the first case the mass lesion was firstly detected at SBCE. In the second patient, a segmental bowel thickening with contrast enhancement was described at magnetic resonance enterography and DBE confirmed a jejunal stenosis. Mucosal sampling of the adjacent mucosa showed atrophic features in both patients (grade 3a and 3b lesions respectively, according to Marsh-Oberhuber classification).[6] An ileal neuroendocrine tumour was identified at SBCE and DBE in the terminal ileum. All the three patients presented with iron deficiency anemia.

Six cases with ulcerative lesions were described. Three of these patients were classified as type II RCD, with monoclonal pattern at TCR gamma rearrangement (Figure 1A). Minute whitish raised patches were observed at enteroscopy in the proximal and mid SB in two type II RCD patients (Figure 1B).

In a patient not adherent to GFD, SBCE showed multiple small erosions in the distal ileum and histological evaluation supported the hypothesis of Crohn's disease, according to the presence of an inflammatory infiltrate deep in the submucosa and focal cryptitis.

In two patients poorly compliant to GFD, a skipped distribution of mucosal atrophy was observed, with involvement of the jejunum and relative sparing of the duodenum. Moreover, an irregular, patchy distribution of atrophic features was described in two GFD treated patients who underwent enteroscopy due to severe gastrointestinal symptoms, after a short period on GFD (four months) (Figure 1C).

Small bowel imaging was performed for diagnostic completion purposes in fifteen cases (computerized tomography and magnetic resonance enterography in nine and six patients, respectively). Indications were a refractory state (n=5), gastrointestinal symptoms (n=6), anemia (n=3), GFD adherence (n=1). Computerized tomography enterography (CTE) was negative in the patient with confirmed ileal neuroendocrine tumour, 1 cm diameter. Four patients presented a jejunoileal fold pattern reversal; three of these patients also underwent SBCE, with the evidence of atrophy-related features extensively involving the SB (more than one third of the SB length). For small bowel findings in single patients see table in Supplemental Digital Content 1.

4.3.4 Follow up

Double-balloon findings induced a modification of patients management in eight cases (33%), as surgery was planned in the three neoplastic cases, steroidal regimen was prescribed in four with persistent gastrointestinal symptoms despite GFD and mucosal atrophy, after having excluded of the presence of lymphoproliferative disorder) at SB sampling; finally, Azathioprine was added in a patient with steroid-dependent RCD.

Patients underwent follow up with regular clinical controls for a median time of 21 months (range 0-60). Two patients died at follow up, due to multi-organ failure in type II RCD and severe cardiac failure, respectively.

4.4 Discussion

Data from present study clearly support the clinical usefulness of DBE to identify SB complications in CD. In fact, DBE enabled us to early diagnose SB tumours (two jejunal adenocarcinomas and an ileal neuroendocrine tumour) as well as to histologically rule out malignancies in mucosal lesions detected at SBCE. This is the first series evaluating the role of DBE in patients with complicated CD. A single study had previously investigated the diagnostic gain of DBE in RCD, and in their selected cohort Hadithi et al.[94] described EATL in patients presenting with multiple SB ulcers and stenosis. Moreover, no malignant complications were detected in this series in the presence of minor endoscopic findings, such as flattened villi, loss of folds, scalloping, nodularity. Ulcerative lesions and stricturing with associated mucosal flattening and nodularity were the reported features of EATL in case reports in which diagnosis was performed by enteroscopic tools.[96],[97] On the other hand, when SBCE was performed in patients with known gastrointestinal lymphoma, slight mucosal alterations (villous atrophy, ulcerations, plaques, white villi) were described.[98] Multiple polyps and mucosal granularity were detected by DBE in the SB of subjects affected by gastrointestinal lymphoma; these "diminutive lesions" were reported to occur mainly in the

follicular and MALT lymphoma histological type.[99] Thus, the comprehension of the clinical predictive value of SBCE findings is presently poor and standardized indications to DAE are lacking, especially as far as an early diagnosis of EATL is concerned.

In our study cohort, DBE was performed for histological definition of lesions detected at SBCE or when capsule endoscopy was contraindicated due to obstructive symptoms. Male sex and older age

were more frequent in the cohort of CD patients with a suspected complicated course in comparison with the "control" population represented by non complicated CD patients on GFD. Age at diagnosis has already been reported as a "risk factor" for a reduced response to GFD, even if data about possible reasons for that, such as gluten exposure, have not been clearly elucidated.[15],[100] Among the patients undergoing DBE in the two referral Centers, the most common indication was the follow up of a known refractory state. Indication to enteroscopy in the three patients with a subsequent diagnosis of SB malignancy was persistent iron deficiency anemia despite ongoing adherence to GFD; therefore this finding should be interpreted as a possible sign of a complicated course. All the three neoplastic patients had a favourable staging, further supported by the curative surgery without need for any additional therapy and lack of recurrences at follow up.

In the patients with type II RCD, i.e. the condition associated with the higher risk of EATL, SBCE detected mucosal alterations (raised patches, ulcerations) and DBE was carried out aiming at ruling out a malignant evolution. Noteworthy, the prognosis of intestinal lymphoma is usually very poor due to the advanced stage at diagnosis, which is the main determinant for the unsatisfactory low therapeutic effectiveness.[101] Accordingly, any effort should be made to realize a prompt recognition of the disease. In this context, until recent times, main limitation was represented by the difficulty in exploring the small bowel. With the availability of the enteroscopic techniques, the present main focus is the identification of the SB mucosal findings unveiling early stage malignant lesions and/or alterations associated with subsequent malignant evolution (premalignant lesions), but the characteristics of these "alarm endoscopic features" are not defined. As a consequence, the selection of the mucosal alterations of the small bowel deserving investigation is now committed to experts judgement. In the patients followed at the two referral Centers involved in this series, in the case of ulcers, protruding mucosal alterations, irregular distribution of atrophy features, a histological study was planned. In the RCD II cases, biopsies from DBE gave negative results for an underlying lymphoproliferative disorder. After exclusion of malignant complications, RCD

diagnosis was confirmed and steroid therapy was prescribed; no cases of intestinal lymphoma developed during follow up.

Ulcerative-type alterations were observed in patients with both polyclonal and monoclonal TCR gamma rearrangement, in line with the low specificity of some endoscopic findings.

In those cases investigated due to poor adherence to GFD or persistence of symptoms while on a still short lasting GFD, endoscopic features of atrophy were described with a patchy distribution. Biopsies during DBE ruled out complications other than atrophy in association with this mucosal pattern. The observation could shed some light on the endoscopic picture of the reversal process taking place in the SB of treated CD patients.

The present study is limited by its retrospective design. However, since evidence-proven criteria for the selection of endoscopic findings deserving DAE are lacking, the experience of two tertiary referral centers is reported. Comparison with a control CD group was only feasible as far as demographical data is concerned; DBE data were obtainable, as is evident, only from cases with presumed complicated course. High number of procedures and heterogeneity of indications mainly derives from present inaccurate definition of SB endoscopic findings suspected for concealing clinically relevant pathological processes.

Overall, in this large series DBE was proven to be useful in selected CD cases to exclude/confirm malignant or premalignant conditions, possibly associated with even minor mucosal lesions. Furthermore, DBE altered clinical management in about one third of patients. "Negative" histological findings resulted also useful to influence therapeutic decisions in a relevant subset of patients. Ongoing research about the identification of clinical factors and endoscopic findings associated with a higher risk of neoplastic evolution in CD will address appropriate indications to DBE and early diagnosis.

5. miRNAs AS POSSIBLE BIOMARKERS OF ENTEROPATHY-ASSOCIATED T CELL LYMPHOMA DEVELOPMENT IN CELIAC DISEASE

5.1 Introduction

Refractory CD is associated with an increased risk of EATL, representing an "intermediate" state between intestinal atrophy and lymphoma. A subset of non-refractory CD patients presents an increased neoplastic risk as well. The identification of "at risk CD subjects" with novel biomarkers could be pivotal in the selection of patients to be carefully managed for the early detection of SB malignancy. This plan is actually hindered by the absence of such a biological marker, except for the analysis of TCR gamma rearrangements for the classification of refractoriness

MicroRNAs (miRNAs) are small noncoding RNAs regulators of gene expression with a role in several immune mediated and neoplastic processes with potential diagnostic, prognostic and therapeutic implications. We previously reported how different clinical phenotypes of CD display unique miRNA signatures.[102] The discovery of EATL molecular biomarkers useful to stratify CD patients according to their neoplastic risk could allow to pursue a "personalized medicine" approach. Our hypothesis is that miRNAs through the regulation of gene expression may play a role in immune response and carcinogenesis in CD, as demonstrated in other inflammatory, autoimmune and neoplastic conditions.[103] Identification of the presence of such biomarkers in association with EATL could shed light on the molecular pathogenesis of this malignancy, therefore improving our understanding of neoplastic transformation of inflamed CD mucosa. Moreover, we expect that the identified miRNA signatures are differently expressed according to different clinical scenario. Since only a limited subgroup of CD patients will develop SB malignant complications, we expect to identify an association between higher neoplastic risk, such as the clinical conditions of refractory disease, and a subset of miRNAs characteristic of CD-related EATL. The identified miRNA signatures could support the identification of other categories of CD patients at risk of

neoplasia who deserve an accurate clinical management. Since no data are available regarding molecular alterations underpinning neoplastic transformation in RCD patients beside TCR gamma rearrangement, this project will provide a novel biomarker predictive of disease aggressiveness to support a timely pharmacological intervention.

Moreover, small bowel investigations have been recently ameliorated by the introduction of enteroscopy and small bowel targeted imaging techniques. These useful diagnostic tools are costly and, often, invasive. Identification of molecular biomarkers associated to the development of intestinal lymphoma in CD could lead to targeted selection of the subset of patients deserving an accurate evaluation of the small bowel and long term surveillance. The final goal will be to obtain a timely diagnosis, a better prognosis and optimization of health resources.

5.2 Methods

5.2.1 Patients

Celiac disease and RCD diagnosis was performed in accordance with the international guideline. EATL diagnosis was based on the criteria proposed by the WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues, Fourth Edition.

Intestinal samples are from surgical resections in cases of malignancies (EATL) or from endoscopic duodenal biopsies (Olympus endoscopes and standard forceps, Boston Scientific) in case of RCD, CD or healthy subjects.

5.2.2 Molecular analyses

miRNA profiles will be obtained from duodenal mucosa, intra-epithelial infiltrating lymphocytes (IEL) or intestinal-associated lymphomas components. Specifically, the aforementioned tissue categories will be purified from archival tissue blocks by laser-assisted microdissection (LMD,

Leica LMD6000, Leica Microsystems, Milan, Italy) as previously described (Savi et al Clinical Science 2014; Vaira et al Oncogene 2012) after identification of the appropriate blocks through morphological and histopathological examination. Following a pre-amplification step, miRNA expression will be profiled by TaqMan® Low Density Array Human MicroRNA Panel technology (Life Technologies, Carlsbad, CA, USA) which enables accurate quantitation of 754 mature miRNAs. miRNAs will be then relatively quantified in each sample on reference sno-RNAs (U6, RNU44 and RNU48) or on the three most stable transcripts using GeNorm and DataAssist software.

When the expression of single miRNAs will be analyzed, specific primers and probe for the selected target and reference transcripts will be used (TaqMan microRNA assays, Life Technologies).

5.2.3 Statistical analyses

miRNAs relative quantities will be normalized and log2 transformed before being imported in BRB Array Tools software (http://linus.nci.nih.gov/BRB-ArrayTools.html). To identify miRNAs specifically associated to CD-EATL, miRNAs will be filtered before being queried by the Class comparison and Class prediction analyses. The capacity to discriminate between CD-associated EATL from other intestinal lymphomas of the single miRNA or of signatures of miRNAs will be then assessed by ROC curves (MedCalc software).

Selected miRNAs expression will be then verified by single qPCR in the other classes of CD patients and ROC curves analysis will be performed to determine specificity and sensibility of the biomarker in identifying CD patients at higher risk of neoplastic development.

5.3 Preliminary results

Twenty-seven EATL tissue specimens from 10 CD and 17 non-CD patients, respectively, as well as five RCD duodenal biopsies were retrieved from archive of Pathology Division.

From each EATL case we isolated through laser-assisted microdissection (LMD, Leica LMD6000) the lymphoma and the associated epithelial component when available. RCD biopsies were extracted in total. Total RNA was then purified using the MasterPure RNA isolation Kit (Epicentre Biotechnologies) and quantified spectrophotometrically. Two cases (one from each EATL group) were excluded for poor RNA quality. Therefore twenty-five EATL as a whole, 9 from CD patients (6 males) and 16 from non-CD patients (5 males) were included in the miRNA profiling study. EATL-associated mucosa was available from 7 CD (of which 4 were males) and 10 non-CD patients (of which 4 were males).

Globally, 400 or 448 human miRNAs out of 760 (53% or 59%, respectively) were evaluable in EATLs or mucosa tissues of CD related and CD unrelated lymphomas and RCD patients, respectively. At unsupervised analysis, miRNA profiles of EATLs did not distinguish patients according to celiac disease presence. When supervised analysis was performed (t statistic corrected for multiple comparisons) a single miRNA resulted upregulated in CD related EATL ($p=0.0002$) and this data was confirmed at single qPCR analysis ($p=0.0012$), claiming for a potential role as biomarker of intestinal T cell lymphoma developing in CD.

When mucosa specimens were evaluated, global miRNA profiling of RCD clearly distinguished this class of patients ($p=0.001$ vs EATL, both CD and non-CD). Moreover, CD patients showed similar expression levels when taken as a single group that included RCD and CD related EATL and compared to non-CD EATL cases ($p=0.001$).

COMMENTS

Small bowel malignancies occurring in CD represent rare complications of a widespread disease. Despite their low incidence, implementing a secondary prevention strategy is of utmost relevance, since the population they can affect is numerous, growing and regularly attending medical attention for follow up reasons. Accurate, expensive and sometimes invasive diagnostic tools are presently available, but the subset of patients deserving such an approach is partly known.

Presented data do not support the need to screen general population and asymptomatic subjects in order to prevent possible lymphomas. As far as treated CD patients suspected to have a complicated course is concerned, endoscopic techniques are useful to detect early intestinal mucosal lesions, provided that a better understanding of the clinical relevance of the endoscopic features is achieved. Standardized indications to deep enteroscopy and histological sampling are required for optimization of health resources.

Identification of molecular biomarkers, such as miRNA signatures associated to the development of intestinal lymphoma in CD could lead to targeted selection of the subset of patients deserving an accurate evaluation of the small bowel and long term surveillance. A timely diagnosis will allow proposed therapies to be tolerated and prognosis of this subgroup of patients to improve.

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TABLES

Table 1. Number and types of Non Hodgkin Lymphoma (NHL) and CD diagnosis in the Varese province from 1999 to 2004

	1999	2000	2001	2002	2003	2004
NHL (n° of patients) M/F, mean age (years)	214 110/104, 63	190 109/81, 60	173 92/81, 64	198 112/86, 61	208 113/95, 64	197 107/90, 63
GI NHL M/F, mean age (years)	54 27/27, 63	45 20/25, 63	30 16/14, 70	36 19/17, 65	32 20/12, 69	40 18/22, 70
B-cell lymphoma (any site) M/F, mean age (years)	193 97/96, 63	168 95/73, 61	154 82/72, 63	179 98/81, 62	187 98/89, 64	165 93/72, 64
GI B-cell lymphoma M/F, mean age (years)	54 27/27, 63	45 20/25, 63	29 16/13, 70	35 18/17, 65	30 19/11, 68	35 15/20, 70
T-cell lymphoma (any site) M/F, mean age (years)	21 13/8, 62	22 14/8, 58	19 10/9, 68	19 14/5, 57	21 15/6, 58	32 14/18, 56
GI T-cell lymphoma M/F, mean age (years)	0	0	1 0/1, 77	1 1/0, 45	2 1/1, 80	5 3/2, 65
Coeliac disease M/F, mean age (years)	54 9/45, 35	48 16/32, 34	29 6/23, 33	36 11/25, 31	59 15/44, 28	100 21/79, 31
Lymphoma in CD patients	1*	0	0	0	0	0

*Ki-1+ large cell GI lymphoma; exclude from data analysis because contemporary diagnosis of CD and lymphoma

Table 2. Expected cases of Gastrointestinal (GI) B-cell lymphoma in CD patients according to the theoretical prevalence of CD in Varese Province

	Expected cases of GI B-cell Lymphomas (% observed)*			
CD theoretical prevalence (number of cases)	RR = 1	RR = 10	RR = 50	RR = 100
1: 200 (4076)	0·19 (0·5)	1·91 (5·0)	9·57 (25·2)	19·15 (50·4)
1: 100 (8152)	0·38 (1·0)	3·83 (10·0)	19·15 (50·4)	38·31 (100·1)
1: 50 (16304)	0·76 (2·0)	7·66 (20·2)	38·31 (100·1)	76·62 (201·6)

* incidence of GI B lymphoma per 100,000/year in Varese Province = 4·7

Table 3. Expected cases of Gastrointestinal (GI) T lymphoma in CD patients according to the theoretical prevalence of CD in Varese Province

	Expected cases of GI T-cell Lymphomas (% observed)*			
CD theoretical prevalence (number of cases)	RR = 1	RR = 10	RR = 50	RR = 100
1: 200 (4076)	0·008 (0·5)	0·08 (5·4)	0·40 (27·1)	0·81 (54·3)
1: 100 (8152)	0·016 (1·0)	0·16 (10·8)	0·81 (54·3)	1·63 (108·7)
1: 50 (16304)	0·032 (2·1)	0·32 (21·7)	1·63 (108·7)	3·26 (217·4)

* incidence of GI B lymphoma per 100,000/year in Varese Province = 0·2

Table 4. Risk per 10,000 coeliac patients per year of developing a gastrointestinal (GI) B-cell NHL according to the theoretical prevalence of CD and the percentage of coeliac associated lymphomas

	Risk according to the percentage of CD associated GI B cell NHL			
CD theoretical prevalence (number of cases)	100%	50%	25%	1%
1: 200 (4076)	93·22	46·61	23·30	0·93
1: 100 (8152)	46·61	23·30	11·65	0·46
1: 50 (16304)	23·30	11·65	5·82	0·23

Table 5. Risk per 10,000 coeliac patients per year of developing a gastrointestinal (GI) T-cell NHL according to the theoretical prevalence of CD and the percentage of coeliac associated lymphomas

	Risk according to the percentage of CD associated GI T cell NHL			
CD theoretical prevalence (number of cases)	100%	50%	25%	1%
1: 200 (4076)	3·68	1·84	0·92	0·03
1: 100 (8152)	1·84	0·92	0·46	0·02
1: 50 (16304)	0·92	0·46	0·23	0·01

Table 6. Characteristics of the celiac patients prospectively evaluated for gastrointestinal tumors

	Entry criteria			**Overall**	**P value**
	Positive imaging	Non-compliance to GFD	Alarm findings		
Patients, N (%)	7 (7)	16 (15)	82 (78)	105 (100)	
Male sex, N (%)	3 (3)	4 (4)	13 (12)	20 (19)	NS
Age at diagnosis, years	46.7±12	18.9±19.7	42.7±15.6	39.3±18.2	<0.0001
Age at entry, years	50.6±13.6	37.3±12.4	48.4±13.8	46.8±14.0	<0.005
CD onset: classic, paucisymptomatic, DH	4, 2, 1	11, 4, 1	58, 22, 2	73, 28, 4	NS
Positive serology at diagnosis, N (%)	7 (7)	16 (15)	70 (67)	93 (86)	P<0.001
Follow up, years	4.1±5.1	8.8±11.7	5.9±7.1	6.2±7.9	NS
Positive serology at entry, N (%)	1 (1)	8 (8)	20 (19)	29 (28)	NS
Hemoglobin at enrolment (g/dL)	13.7±1.6	13.7±1.3	11.8±1.9	11.8±2.6	<0.0001
Duodenal histology at entry:0,1,2,3a,3b,3c [a]	3,-,-,2,-,-	-,2,1,5,5,2	23,2,2,22,15,2	26,4,3,29,20,4	NS
RCD, N (%)	1 (11)	0	11 (10)	12 (11)	

CD, celiac disease; DH dermatitis herpetiformis; RCD, refractory celiac disease.

Data are presented as mean±standard deviation; percentages refer to overall number of patients.

[a] According to the Marsh-Oberhuber classification.

Table 7. Characteristics of the 24 celiac patients evaluated with double-balloon enteroscopy due to suspected complicated course

	Entry criteria					
	RCD	**GI symptoms**	**Sideropenic anemia**	**Non compliance to GFD**	**Overall**	**P value**
Patients, n	9	6	6	3	24	
Sex, F/M	1/8	4/2	5/1	2/1	12/12	
BMI (kg/cm^2)	20 ± 4	19 ± 2	26 ± 5	23 ± 2	21 ± 4	0.07
Age, years: at diagnosis	44 ± 19	40 ± 21	25 ± 14	30 ± 15	37 ± 20	0.26
at entry	55 ± 13	46 ± 19	41 ± 15	39 ± 8	47 ± 15	0.25
CD onset: classic, paucisymptomatic, DH	9, -, -	5, 1, -	1, 5, -	1, 2, -		
Positive serology at diagnosis, n	6	5	6	3		
Follow up, years	11 ± 23	6 ± 13	12 ± 15	9 ± 15	10 ± 17	0.34
Oral/anal DBE	9/2	2/5	3/3	2/1	16/11	

RCD=refractory celiac disease; GI=gastrointestinal; GFD=gluten free diet; BMI=body mass index; CD=celiac disease; DH=dermatitis herpetiformis; DBE=double-balloon enteroscopy

FIGURES

Figure 1. Celiac disease (CD) related and CD unrelated enteropathy associated T cell lymphoma (EATL) miRNA profiling: supervised analysis, scatterplot (A) and single qPCR analysis of the identified miRNA, *under patent* (Mann Whitney test, p=0.0012) (B).

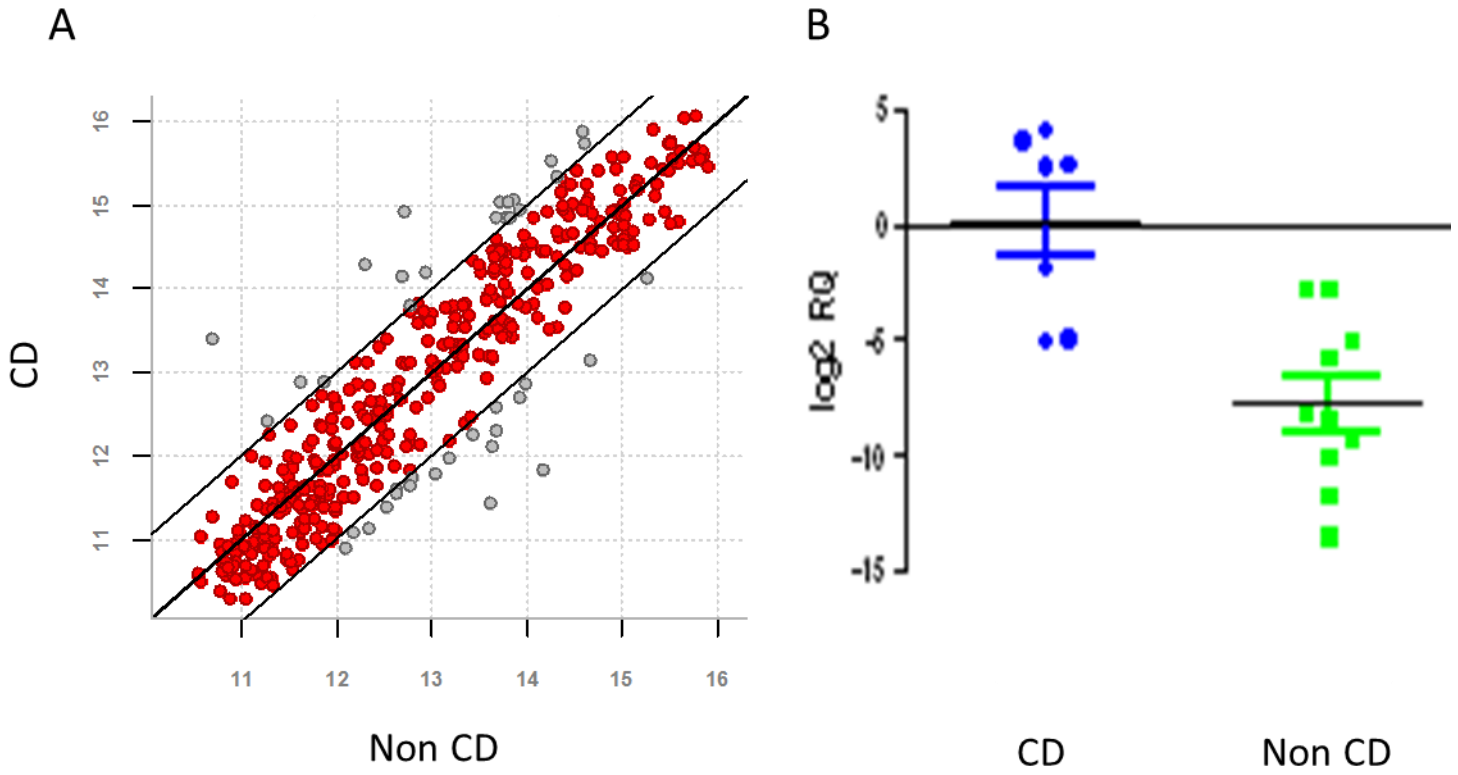

Figure 2. Mucosa miRNA profiling, supervised analyses, class comparison between three groups: celiac disease (CD) related enteropathy associated T cell lymphoma (EATL), CD unrelated EATL, refractory celiac disease (RCD). Global miRNA profiling of RCD clearly distinguished this class of patients (p=0.001 vs EATL, both CD and non-CD)