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Alessandro Carugi
*University of Siena*

Anna Onnis
*University of Siena*

Giuseppina Antonicelli
*University of Siena*

Benedetta Rossi
*University of Siena*

Susanna Mannucci
*University of Siena*

***See next page for additional authors***

**Authors**
Alessandro Carugi, Anna Onnis, Giuseppina Antonicelli, Benedetta Rossi, Susanna Mannucci, Anna Luzzi, Stefano Lazzi, Cristiana Cristiana Bellan, Gian Marco Tosi, Shahin Sayed, Giulia De Falco, and Lorenzo Leoncini

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**Original Research Article**

# Geographic variation and environmental conditions as cofactors in *Chlamydia psittaci* association with ocular adnexal lymphomas: a comparison between Italian and African samples

Alessandro Carugi[1], Anna Onnis[1], Giuseppina Antonicelli[1], Benedetta Rossi[1], Susanna Mannucci[1], Anna Luzzi[1], Stefano Lazzi[1], Cristiana Bellan[1], Gian Marco Tosi[2], Shaheen Sayed[3], Giulia De Falco[1] and Lorenzo Leoncini[1]*

[1]*Department Human Pathology and Oncology, University of Siena, Italy*
[2]*Department of Ophthalmology and Neurosurgery, University of Siena, Italy*
[3]*Aga Khan Hospital, Nairobi, Kenya*

**Correspondence to:*
*Lorenzo Leoncini, Department of Human Pathology and Oncology, University of Siena, Via delle Scotte, 6, 53100 Siena, Italy.*
*E-mail: leoncinil@unisi.it*

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## Abstract

**A particular extra-nodal lymphoma type arises from B cells of the marginal zone (MZ) of mucosa-associated lymphoid tissue (MALT). The aetiology of MZ lymphomas suggests that they are associated with chronic antigenic stimulation by microbial pathogens, among which *Helicobacter pylori*-associated gastric MALT lymphoma is the best studied. Recently, MALT lymphomas have been described in the context of chronic conjunctivitis, which can be associated with *Chlamydia* spp. infection. Studies from Italy showed the presence of *Chlamydia psittaci* in 87% of ocular adnexal lymphomas (OAL), and *C. psittaci* has been described in a large part of samples from Austria and Korea as well. However, this finding was not always confirmed by other studies, suggesting that the association with *C. psittaci* may depend on geographic heterogeneity. Interestingly, none of the studies up to now has been carried out in the African population, where a strong association between infectious agents and the occurrence of human neoplasms has been reported. This study was designed to investigate the possible association of *Chlamydia psittaci* in cases retrieved from Kenya, compared to cases from Italy. Our results showed that there was a marked variation between the two geographical areas in terms of association with *C. psittaci*, as 17% (5/30) of the samples from Italy were positive for *C. psittaci*, whereas no association with this pathogen was observed in any of the African samples (0/9), suggesting that other cofactors may determine the OAL occurrence in those areas. OAL cases are often characterized by down-regulation of p16/INK4a expression and promoter hypermethylation of the *p16/INK4a* gene. Our results showed a partial methylation of *p16/INK4a* promoter in *C. psittaci*-negative cases, whereas no hypermethylation of this gene was found in *C. psittaci*-positive cases, suggesting that mechanisms other than promoter hypermethylation lead to *p16/INK4a* silencing in *C. psittaci*-positive cases. We may conclude that the role of epidemiologic, environmental and genetic factors, must be considered in the aetiology of this disease.**


**Keywords:** **Chlamydia; ocular lymphoma**

## Introduction

Extra-nodal marginal zone B cell lymphoma of mucosa-associated lymphoid tissue (MALT lymphoma) arises in a number of extra-nodal sites, including the gastrointestinal tract, salivary and thyroid glands, lung, ocular adnexa and skin. Interestingly, these organs are devoid of native lymphoid tissue, indicating that lymphoma at these sites arises from MALT acquired as a result of a chronic antigenic stimulation by microbial pathogens or autoimmune disorder [1]. This notion is supported by the observation that a proportion of gastric and skin MALT lymphomas are curable by bacterial eradication alone [2]. In addition, analysis of the Ig variable (IgV) genes also supports the concept of antigen-driven lymphomagenesis in MALT lymphomas. The IgV heavy and light chain genes of these malignancies are heavily mutated, suggesting a germinal centre (GC) or a post-germinal centre derivation, and the mutation patterns unequivocally indicate that antigen-based selection occurs at some stage of their development [3,4]. The inflammatory disease associated with MALT lymphoma not only provides a micro-environment that is crucial for malignant transformation, but the immunological response generated during the inflammatory process also promotes the growth of the lymphoma cells.

The best-characterized model of 'indirect lymphomagenesis' involving sustained stimulation of the immune system linked to malignant transformation is

the *H. pylori*-associated MALT lymphoma of the stomach [5]. Other pathogens have been reported to be associated with the development of MALT lymphomas, although the causative role is not so well established [5].

Ocular adnexal lymphoma (OAL) is a subtype of non-Hodgkin's lymphoma, which most commonly affects MALT, accounting for 60–80% of all OAL cases [6,7]. OAL represents a significant proportion (approximately 12%) of all MALT lymphomas [3], and it is the most common lymphoma of the ocular adnexa [8–10], occurring principally in the conjunctiva, orbital soft tissue and lacrymal apparatus. MALT lymphoma may be associated with chronic conjunctivitis, resembling what happens for gastric MALT lymphoma, which is associated with *H. pylori*-derived chronic gastritis. Interestingly, there is a considerable overlap in both the histological and clinical presentations of chronic conjunctivitis and OAL [11–13]. OAL may arise from MALT acquired as a result of chronic inflammatory responses. The aetiology of OAL is currently unclear, even though it is becoming more and more evident that infectious agents underlying chronic eye infection, as *Chlamydia*, herpes simplex and adenovirus may play a role in ocular lymphomagenesis.

In the past few years, several studies have reported the possible role of *Chlamydia psittaci* in the development of OAL. The first evidence of such an association came from a study performed on an Italian cohort of patients, where *C. psittaci* DNA was detected in 87% of the cases of OAL [14]. The same group reported later the success in eradicating, partially or completely, the disease after *Chlamydia psittaci*-eradicating antibiotic therapy [15]. Since then, many groups worldwide have investigated the possible association between *C. psittaci* and OAL occurrence, but with discordant results [14–25]. This raises the possibility that *C. psittaci* may be variably associated with OAL in different geographic regions and that other aetiological factors may be involved in the development of this lymphoma. It must be considered in fact that the genetic background of different populations, as well as epidemiological risk factors, may vary among different geographic areas and may affect the incidence of lymphomas [26–28]. In particular, the association with *C. psittaci* was reported mostly in subjects coming from rural areas, with a prolonged contact with household animals [14].

Based on the hypothesis that a geographic difference for *C. psittaci* association and OAL exists, we decided to analyse OAL cases from African regions, where the presence of *C. psittaci* in OAL cases has not been investigated to present. The living conditions of this underdeveloped country constitute a fertile *humus* for the association of infectious agents with many pathologic disorders.

Our results demonstrated the lack of association between *C. psittaci* and OAL in all of the Kenyan samples analysed, whereas *C. psittaci* DNA was detected in tumour samples of Italian patients, even though in a lower percentage than that reported in previous studies [14]. Fluorescence *in situ* hybridization (FISH) analysis revealed that chromosomal alterations were present only in a small percentage of the samples, all belonging to the Italian cohort of patients.

The inactivation of the *INK4/p16* gene promoter by hypermethylation seems to play an important role in the development of gastric MALT lymphomas [29–32]. To present, no information is available about the methylation status of this gene in ocular MALT lymphomas. Therefore, we analysed whether hypermethylation of this gene was present in OAL. Our results indicated that p16 was hypermethylated mostly in *C. psittaci*-negative cases, indicating that mechanisms other that p16 epigenetic silencing occur in the presence of the pathogen, and are responsible for cell cycle deregulation.

## Materials and methods

### Case selection, histology and immunophenotype

Cases of lymphoma involving the ocular adnexa were identified from the case files of the Department of Human Pathology and Oncology, University of Siena, Italy ($n = 30$) and the Department of Pathology of the Aga Khan Hospital, Kenya ($n = 9$). Informed consent to carry out the study was granted by the institutions' ethical review committee and it was in accordance with the declaration of Helsinki. Sections made from these paraffin embedded tissue blocks were stained with haematoxylin and eosin to confirm the previous diagnoses. Cases were diagnosed according to the criteria of the WHO classification [3]. Immunophenotype was determined using a panel of antibodies, as previously described [33]. Results of staging studies, including physical examination, computed tomography scan, bone marrow biopsies and bone marrow immunophenotyping, were retrieved when available. Frozen samples from two patients were also used as a control in all of the experiments carried out.

### Chlamydia psittaci detection

For *C. psittaci* detection, DNA was extracted from paraffin-embedded sections using the QIAamp Tissue kit (Qiagen, Hilden Germany), following manufacturer's instructions, and assessed by polymerase chain reaction (PCR). The presence of *C. psittaci* in all of the samples was investigated by TETR-PCR, following the protocol described by Madico *et al* [34]. More in detail, one primer is located in the 16S rRNA gene and the other in the 16S–23S spacer region, as reported [16]. DNA of *C. psittaci*, used as a positive control, was a kind gift of Dr Dolcetti (Aviano, Italy). PCR products were visualized by gel electrophoresis and specificity of amplicons was confirmed by direct sequencing of purified PCR products on an ABI PRISM 310 (Applied Biosystems, USA).

### Methylation-specific PCR (MSP) assay

PCR of genomic DNA extracted from FFPE primary tumours was performed for methylation analysis of the *INK4a/p16* gene promoter. Primer sequences were retrieved from the literature [34]. The assay is based on the DNA sequence differences between methylated and



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unmethylated DNA after bisulfite modification by EpiTect Bisulfite Kits (Qiagen). The bisulfite reactions were performed according to the manufacturer's instructions. MSP with primers specifically designed for discriminating between methylated and unmethylated (Tm 65°C) was performed (Table 3). PCR products were analysed on 2% agarose gel.

### Fluorescence *In Situ* Hybridization (FISH)

FISH analysis for the detection of the most common re-arrangements occurring in OAL was performed by applying a large panel of probes (Table 1), following standard protocols used during EUROFISH and available at www.euro-fish.org. Briefly, paraffin tissue sections (4 μm) were deparaffinized, air-dried, immersed in a jar filled with pre-treatment solution and warmed at 98°C for 10 min by means of a Whirlpool JT 356 microwave (Dako). Subsequently, the slides were cooled for 15 min at RT. After two passages in Wash Buffer, 3 min each, excess buffer was taped off and the slides digested with cold Pepsin for 20 min in a Dako Cytomation Hybridizer. The slides were then washed twice in Wash Buffer for 3 min, dehydrated using increasing graded ethanol series, air-dried and finally 10 μl of Probe mix were applied to each tissue section. The slides, covered with coverslip and sealed with rubber cement, were then incubated in the DakoCytomation Hybridizer (Dako) according to manufacturer's recommendation. Next day, slides were treated with stringency buffer at 65°C for 2 min, then placed twice in Wash Buffer for 3 min, dehydrated using increasing graded ethanol series, air-dried, and counter-stained applying 15 μl of Fluorescence Mounting Medium. Hybridization signals were visualized using a Leica microscope equipped with a triple-band filter for detecting green fluorescent protein (GFP)/spectrum green, Texas red/spectrum orange, and DAPI/spectrum blue. Images were captured and archived using Leica FW4000 software. One hundred non-overlapping interphase nuclei were scored for each tumour specimen. In normal nuclei, two yellow fusion signals (2F) are detected, whereas in nuclei with translocations, a yellow (or red-green juxtaposed) signal is accomplished by one red and one green segregated signal (1F1R1G). All reagents and probes were purchased from DakoCytomation Glostrup, Denmark.

**Table 1.** Probes used for FISH analysis

| Probe | Locus |
|---|---|
| BCL10-split signal (DAKO) | 1p22 |
| CCND1-split signal (DAKO) | 11q13 |
| MALT1-split signal (DAKO) | 18q21 |
| IGH-split Signal (DAKO) | 14q32 |
| IGL-split signal (DAKO) | 22q11 |
| BCL6-split signal (DAKO) | 3q27 |
| BCL2-split signal (DAKO) | 18q21 |
| MYC-split signal (DAKO) | 8q24 |

## Results

### Clinical, morphological features and immunophenotype

The main characteristics of patients are reported in Table 2. Cases were diagnosed, based on morphology, immunophenotype and genetic alterations, in accordance to the WHO classification, as follicular lymphomas (FL) (3),

**Table 2.** Clinical information of the cases

| GP | Diagnosis | Stage | Sex/Age | FISH | Chlamydia | IHC | p16-M |
|---|---|---|---|---|---|---|---|
| IT | FL | IV | F/47 | t(14;18) | − | − | + |
| IT | FL | IV | F/75 | t(14;18) | − | +/− | − |
| IT | FL | IV | F/64 | t(14;18) | − | − | + |
| IT | DLBCL | II | M/58 | — | − | + | − |
| IT | DLBCL | III | M/88 | — | − | − | + |
| IT | MCL | II | M/70 | t(11;14) | + | − | − |
| IT | MCL | I | M/78 | t(11;14) | − | − | + |
| IT | MCL | II | F/61 | t(11;14) | − | +/− | +/− |
| IT | MZL | I | F/36 | — | − | − | +/− |
| IT | MZL | I | M/58 | — | + | − | − |
| IT | MZL | I | M/77 | — | − | − | − |
| IT | MZL | I | M/65 | | − | + | − |
| IT | MZL | I | F/64 | t(11;18) | − | +/− | − |
| IT | MZL | I | F/64 | — | − | − | +/− |
| IT | MZL | I | M/79 | — | − | − | − |
| IT | MZL | I | F/61 | — | − | +/− | − |
| IT | MZL | I | F/63 | — | − | − | − |
| IT | MZL | I | M/64 | — | − | +/− | − |
| IT | MZL | I | F/67 | — | − | − | − |
| IT | MZL | I | M/58 | t(11;18) | − | + | − |
| IT | MZL | I | M/34 | — | − | +/− | − |
| IT | MZL | I | F/76 | — | − | − | − |
| IT | MZL | I | F/68 | t(11;18) | − | − | +/− |
| IT | MZL | I | F/72 | — | − | + | − |
| IT | MZL | I | F/76 | — | − | − | − |
| IT | MZL | I | M/79 | — | + | + | − |
| IT | MZL | I | M/52 | — | − | − | − |
| IT | MZL | I | M/61 | — | + | +/− | − |
| IT | MZL | I | M/46 | — | + | − | − |
| IT | MZL | I | M/48 | — | − | − | − |
| KE | MZL | I | F/53 | — | − | +/− | − |
| KE | MZL | I | F/51 | — | − | − | +/− |
| KE | MZL | I | M/47 | nv | − | − | +/− |
| KE | MZL | I | M/41 | — | − | + | +/− |
| KE | MZL | I | F/43 | — | − | − | +/− |
| KE | MZL | I | M/51 | nv | − | +/− | +/− |
| KE | MZL | I | M/70 | — | − | − | + |
| KE | MZL | I | F/35 | nv | − | − | + |
| KE | MZL | I | F/70 | — | − | − | + |

IHC, Immunohistochemistry detecting p16/INK4a expression; P16-M, Methylation status of the *p16/INK4a* gene promoter; GP, Geographic provenience of the cases; IT, Italian; KE, Kenyan.

**Table 3.** Primers used for MSP of *p16/INK4a* gene promoter

| Probe | Locus |
|---|---|
| M-*p16/INK4a* | 5'-TTATTAGAGGGTGGGGCGGATCGC-3' |
| | 5'-GACCCCGAACCGCGACCGTAA-3' |
| U-*p16/INK4a* | 5'-TTATTAGAGGGTGGGGTGGATTGT-3' |
| | 5'-CAACCCCAAACCACAACCATAA-3' |

M-*p16/INK4a*: methylated DNA.
U-*p16/INK4a*: unmethylated DNA.



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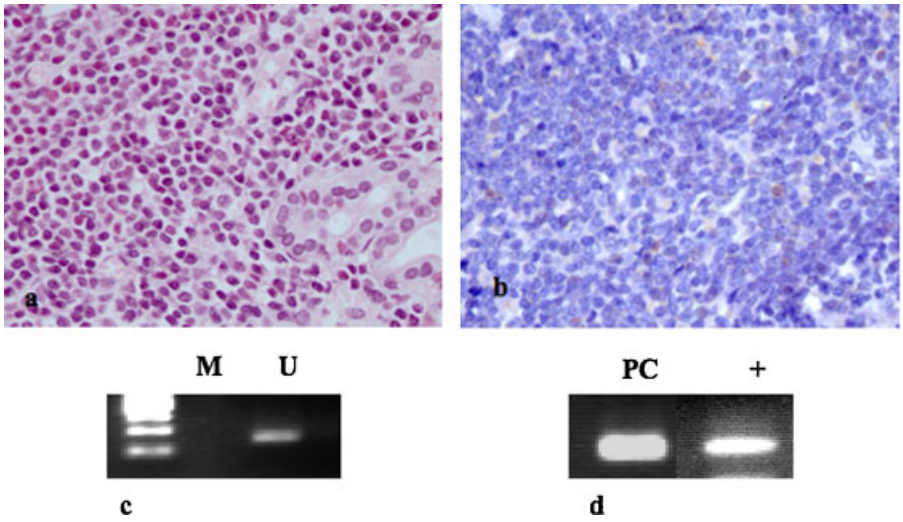


**Figure 1.** (a–d): (a) Hematoxilin/eosin staining of a *C.psittaci*-positive OAL case; (b) immunostaining for p-16/INK4a; (c) methylation analysis of the *p16/INK4a* gene promoter. No amplification is detected using specific primers for methylated DNA (M), whereas a product is detected upon amplification with specific primers for unmethylated DNA (U), indicating that the *p16/INK4a* gene promoter is not methylated; (d) TETR-PCR for *C. psittaci* detection: PC, Positive Control, + indicates the positivity to the pathogen of the OAL sample

diffuse large B cell lymphomas (DLBCL) (2), mantle cell lymphomas (MCL) (3) and marginal zone lymphomas (MZL) (30), representing the vast majority. The male/female ratio was 1:1. The age range was 34–79 (Table 2).

## FISH analysis

FISH analysis confirmed the diagnoses of FL and MCL, by detecting the typical translocations t(14;18) and t(11;14), respectively. Translocation t(11;18), which is frequently detected in MZL, was found only in three cases.

## *C. psittaci* detection

DNA extracted from OAL primary tumours was analysed for the presence of *C. psittaci*, as described in Materials and methods section. Table 2 summarizes our results. *C. psittaci* was not detected in any of the African samples (0/9), whereas 17% of the Italian samples (5/30) were positive for the pathogen. Interestingly, one case of MCL resulted positive for Chlamydia, indicating that Chlamydia infection may not be exclusively restricted to MZL.

## p16/INK4a expression in ocular adnexal lymphoma cases

We then checked the expression of p16/INK4a, at the protein level, by immunohistochemistry, in both *C. psittaci*-positive and negative cases. Our results are reported in Table 2 and indicate that p16/INK4a is mostly not expressed in OALs, independently of *Chlamydia* infection. In addition, both African and Italian cases, showed the same expression pattern for p16/INK4a (Table 2), (Figures 1 and 2).

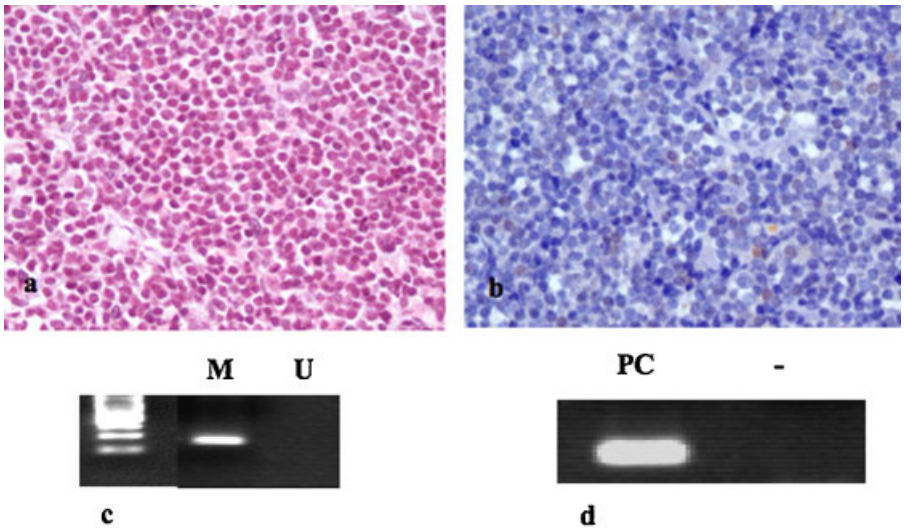


**Figure 2.** (a–d): (a) Hematoxilin/eosin staining of a *C. psittaci*-negative OAL case; (b) immunostaining for p-16; (c) methylation analysis of the *p16/INK4a* gene promoter. Amplification is detected using specific primers for methylated product (M), whereas no product is detected upon amplification with specific primers for unmethylated DNA (U), indicating that the *p16/INK4a* gene promoter is hypermethylated; (d) TETR-PCR for *C. psittaci* detection: PC, Positive Control, – indicates the negativity to the pathogen of the OAL sample



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### Methylation of p16/INK4A in ocular MALT lymphomas

To investigate the molecular mechanism underlying *p16/INK4a* silencing, we analysed the methylation status of the *p16/INK4a* gene promoter, both in *C. psittaci*-positive and negative cases, in all of our cases (Figures 1 and 2). Interestingly, we found a marked difference in terms of *p16/INK4A* methylation between Italian and African samples, being methylated for this gene promoter 27% of Italian cases (8/30), versus 100% (9/9) of the African samples (Table 2). Intriguingly, methylation of *p16/INK4A* was always observed in *C. psittaci*-negative samples in both casistics (Figure 2 c–d). Notably, as *C. psittaci*-positive cases showed no promoter hypermethylation for the *p16/INK4a* gene, even though this gene was not expressed at the protein level, mechanisms other than epigenetic silencing, may be responsible for *p16/INK4a* down-regulation in the presence of *C. psittaci*.

## Discussion

OALs constitute less than 1% of all non-Hodgkin's lymphoma and account for approximately 8–20% of all extra-nodal lymphomas [35]. The vast majority of the OALs are primary extra-nodal marginal zone B-cell lymphomas of MALT-type. MALT lymphoma arises in a number of extra-nodal sites, which are devoid of native lymphoid tissue, indicating that lymphoma at these sites arises from MALT acquired as a result of a chronic antigenic stimulation[1,3,4]. The best-characterized model of 'indirect lymphomagenesis' involving sustained stimulation of the immune system linked to malignant transformation is the *H. pylori*-associated MALT lymphoma of the stomach [5]. Other pathogens have been reported to be associated with the development of MALT lymphomas, although the causative role is not so well established [5].

In this study we focused on the possible role of *C. psittaci* in the pathogenesis of OAL, as its aetiology is currently unclear, but it is becoming more and more evident that infectious agents underlying chronic eye infection, as *Chlamydia*, herpes simplex and adenovirus may play a role in ocular adnexal context [18,26]. Different studies have reported the possible role of *C. psittaci* in the development of OAL, ranging from its association in 87% of the cases in a cohort of Italian patients, to no association, as reported by studies from the US, Netherland, Japan [20–22,36]; these findings suggest that there is a geographic variability for *C. psittaci* association and that other aetiological factors may be involved in the development of this lymphoma.

In this paper we report for the first time the results of a study performed on an African population, where underdeveloped living conditions and the *plethora* of infectious agents may constitute fertile *humus* for malignant transformation. Interestingly, no association with *C. psittaci* was observed in the African cases, suggesting that mechanisms other than *Chlamydia* infection may be responsible for the disease occurrence in these regions. It is worth noting that the genetic background of different populations may give an explanation for this variability, as genetic polymorphisms may be responsible for either resistance or susceptibility to a particular infectious agent. In addition, the exposure to different epidemiological risk factors may vary among different geographic areas and may affect the incidence of lymphomas [27,28].

On the other hand, our results confirm the association with *C. psittaci* in the Italian population, even though at a lower percentage than that previously described.

The finding that one case of MCL was positive for *C. psittaci* infection suggests that the presence of this pathogen may not be exclusive for MALT lymphomas, even though we cannot rule out the possibility that Chlamydia infection and lymphoma occurrence may not be linked in such case.

FISH analysis performed on our samples revealed that chromosomal alterations characteristics of MALT lymphoma were present only in a small percentage of the samples, all belonging to the Italian cohort of patients. Intriguingly, the few translocated cases were negative for *C. psittaci*, resembling what already observed in gastric MALT lymphomas, where translocations are predominantly observed in *H. pylori*-independent cases [24,37–39].

Hypermethylation of CpG islands within promoter regions of genes is associated with transcriptional inactivation and represents an important mechanism of gene silencing in the pathogenesis of malignancies. This epigenetic phenomenon acts as an alternative to mutations and deletions for disrupting the gene function. Methylation of a large number of genes has been described in virtually all types of cancers. MALT lymphomas show a high grade of *p16/INK4a* methylation [29–32,40,41], suggesting that inactivation of *p16/INK4a* by methylation may play an important role in the development of MALT lymphomas. In particular, it has been suggested that methylation of this gene is one of the early events in the development of lymphoid malignancies [42–44]. We analysed the methylation status of *p16/INK4a* in relation with Chlamydia infection, and our results showed that it was hypermethylated mostly in *C. psittaci*-negative cases, indicating that mechanisms other that *p16/INK4a* epigenetic silencing occur in the presence of the pathogen and are responsible for cell cycle deregulation.

Collectively, our results confirm the geographic variability for the association of *C. psittaci* and the onset of OAL, suggesting that other environmental, genetic and epidemiological factors may be relevant for lymphomagenesis. It is imperative to further explore the molecular mechanisms underlying this pathogenesis to both improve diagnosis and design tailored therapeutic approaches.

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