RESEARCH ARTICLE

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Assessment of the Association of *Chlamydia e pneumoniae* Infection with Lung Cancer in a Moroccan Patients' Cohort

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Abstract

Background: *Chlamydia pneumoniae* (*C. pneumoniae*) is a respiratory pathogen associated with chronic inflammatory and its detection in human lung cancer suggests its involvement in cancerogenesis. Our study aimed to evaluate the association between *C. pneumoniae* infection and Lung Cancer disease in Moroccans patients and control cohorts, through a molecular investigation. **Methods:** The study comprised 42 lung cancer patients and 43 healthy controls. All participants provided demographics, Clinical, and Toxic behaviors datas, and a peripheral blood sample for testing, a Nested Polymerase Chain Reaction (PCR) was performed for *C. pneumoniae* Deoxyribonucleic acid (DNA) detection. Statistical analysis was performed using IBM®SPSS®software. **Results:** Positive Nested PCR results for cases and controls were respectively 33.3% and 4.7%, there by significant difference between cases and controls infection was identified ($p < 0.05$). Data analysis also showed that tobacco could act synergically with C. pneumoniae infection as a risk factor of lung cancer. In fact a significant difference between patients and controls was shown for tobacco and alcohol use ($p < 0.05$). **Conclusion:** *C. pneumoniae* infection is potentially associated with primary Lung cancer in the Moroccan population and has combined effects with Tabaco consumption.

Keywords: Chlamydia pneumoniae- infection- case-control study- risk factors- lung cancer

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Introduction

Lung cancer (LC) is the most severe and commonly diagnosed cancer globally. In 2020, approximately 2.21 million new LC cases were diagnosed worldwide, with an estimated 1.79 million death cases (WHO, 2020). In Morocco LC constitutes 12.4% of diagnosed cancer with a mortality rate of 18.6% (WHO, 2020).

Tobacco consumption remains the main etiological risk factor to LC. Other factors such as genetic susceptibility, low diet and air pollution can act independently or together as cofactors with smoking leading to LC (Malhotra et al., 2016).

Furthermore, the involvement of the microbiological agents in the development of cancers diseases was reported in many investigations, indeed multiple earlier studies demonstrated increased menace of LC in Human immunodeficiency virus (HIV) infected patients (Webel et al., 2021; Garcia et al., 2020; Siegel et al., 2012), in the other hand, multiple studies linked infections with Mycobacterium tuberculosis and LC (Cheon et al., 2020; Keikha and Esfahani., 2018). Recently, the study of Alshamsan et al predicted the possible involvement of Chlamydia pneumoniae (*C. pneumoniae*) infection in LC etiology (Alshamsan et al., 2017). Furthermore, a meta-analysis including thirteen publications involving 2,549 LC patients and 2,764 healthy controls revealed that *C. pneumoniae* infection was considerably correlative with

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LC, effectively twelve studies described the association between serological *C. pneumoniae* immunoglobulin's A (IgA) and LC risk; The pooled results indicated that the *C. pneumoniae* infection significant increased the risk of LC OR = 2.07 (95% confidence interval [CI]: 1.43–2.99). And between serological *C. pneumoniae* immunoglobulin's G (IgG) and higher risk of LC; the pooled results indicated that the *C. pneumoniae* infection significant increased the risk of lung cancer OR = 2.22 (95% confidence interval [CI]: 1.41–3.50) (Hua-Feng et al., 2015).

C. pneumoniae is an intracellular Gram-negative bacterium, initially described as a pathogen to humans and animals, causing respiratory tract diseases (Ozturk et al., 2021) and suspected of being partly responsible for the development of cardiovascular diseases (Ozturk et al., 2021; Di Pietro et al., 2019; El Yazouli et al., 2017).

The development of *C. pneumoniae* infection begins when the elementary body (EB), metabolically inactive enters the breathing tract via inhalation and then enters the cells via receptor-mediated endocytosis, differentiating right into a reticulate frame (RB) inside the inclusion. RBs are metabolically active and able to editing host pathways, after approximately 48 to 72 hours, the RBs are ultimately launched as EBs outside the host cells by cellular lysis and infect the environmental cells continuing the infectious process. The organism can get away the endocytic-lysosomal pathway of host cells to stay chronic within tissues below disturbing situations and reactivate while favorable circumstance (Gautam and Krawiec., 2022).

Our study aimed to evaluate the association between *C. pneumoniae* infection and LC disease in Moroccans patients and control cohorts, through a molecular investigation.

Materials and Methods

Sampling

We carried out a case-control study, where a total of 42 patients and 43 controls were investigated for the presence *C. pneumoniae* infection. Adult patients suffering from LC were admitted respectively from two different Hospitals in Rabat, Morocco: Instruction hospital of Mohammed V, of Rabat, and Moulay Youssef Hospital of Rabat. All cases had pathological confirmation of primary LC and had not received any immunosuppressor therapies before the blood samples were taken. In addition, the group of healthy controls was collected from the Transfusion Blood Center (TBC) of Rabat.

Ethics

All participants were volunteers and provided written informed consent. This study was approved by Ethics Committee for Biomedical Research in the Faculty of Medicine and Pharmacy of Rabat (CERB), (IORG Number: IORG0006594) Morocco. The committee's reference number: 38/13.

DNA isolation and molecular screening

Genomic DNA samples were extracted by the High pure isolation kit for genomic nucleic acid (Roche, High pure Pcr template preparation kit. USA 11796828001), following the manufacturer recommendations. Then, a nested Polymerase Chain Reaction (PCR) was performed for screening, using HL1 (5′-GTTGTTCATGAAGGCCTACT-3′)/HR1 (5′-TGCATAACCTACGGTGTGTT-3′) as outer primers and IN1 (5′-AGTTGAGCATATTCGTGAGG-3′) / IN2 (5′ -TTTATTTCCGTGTCGTCCAG-3′) as inner primers, which target a 128 bp fragment (Maass et al., 1998). For both PCR steps, DNA samples were amplified in 25 µl volume, containing 0.5 µM of primers, 0.2 mM deoxyribonucleotides, 1X PCR buffer, 1.5 U / µl Taq polymerase (Invitrogen, Innis, MA et al., (1998) Proc. Natl. Acad. Sci. USA 85, 9436.), 2.5 Mm $Mgcl_2$, 2.5µl DNA sample, and 14.25 of H_2o. Each PCR assay was run with positive and negative controls. The PCR was performed in the BIORAD thermocycler (S1000 ™ Thermal Cycler) with cycling conditions for both rounds as follows: one cycle at 95°C for 5 min and 35 cycles at 94°C for 30 s, 60°C for 30 s and 72°C for 1 min, followed by strand elongation at 72°C for 10 min, then the PCR products were visualized by electrophoresis on 1.5% agarose gel (EL Yazouli et al., 2018).

Statistical analysis

All data were analysed using IBM®SPSS®software version 22. Quantitative variables were compared by Student t-test, fisher's exact test was used to compare qualitative variables and PCR results. P-values less than 0.05 were considered statistically significant.

Furthermore, univariate and multivariate analysis were performed to assess the relationship between LC and *C. pneumonia* infection looking its role as a risk factors.

Results

Characteristics of the study population

The main characteristics of the 85 individuals enrolled in this study revealed that, the majority of the participants were male gender, the mean ages of the studied groups were respectively 56.52±6.06 [37– 70] years for the patients and 54.16±6.73 [35– 64] years for healthy controls, and most participants were Arabs for (Table 1).

The analysis of the collected data also revealed that 88.1% of patients were more likely to be smokers, 31% to drink alcohol and, 23.8% to consume cannabis; Compared to healthy participants the difference is statically significant for tobacco and alcohol ($P<0.05$) (Table 2). The clinical distribution of LC symptoms showed a dominance of caught (76.2%) followed by dyspeniea (59.5%), chest pain (57.1%), weight loss (54.8%), haemoptysis (40.5%), and fever (11.9%) (Table3). Furthermore, adenocarcinoma was the predominant histological type of LC (40.5%) followed by squamous cell carcinoma (38.1%), Non-Small cell lung cancer (14.3%), and finally Small cell lung cancer (7.1%), (Table 3). The major part of the patients was diagnosed with stage III or IV. Control group subjects were clinically safe and didn't show clinical signs of primary lung cancer.

Table 1. Socio-Demographic and Risk Factor Characteristics of Cases and Control

Characteristics	Case (n= 42)	Control (n= 43)	P-value
Age (years)±Mean [min– max]	56.52±6.06 [37– 70]	54.16±6.73 [35– 64]	0.093
Age group (year)			
≤45 (%)	1 (2.39)	5 (46.51)	0.202
46-55 (%)	14 (3.33)	16 (37.21)	0.821
56-65 (%)	25 (59.52)	22 (51.16)	0.515
>65 (%)	2 (4.76)	0	0.241
Gender			
Male (%)	41 (97.6)	39 (90.7)	0.360
Female (%)	1 (2.4)	4 (9.3)	0.360
Ethnic origin			
Arab (%)	24 (57.1)	29 (67.4)	0.375
Amazigh (%)	4 (9.5)	4 (9.3)	1.00
Sahraoui (%)	0	5 (11.6)	0.055
Soussi (%)	3 (7.1)	1 (2.3)	0.360
Jebli (%)	2 (4.8)	0	0.241
Arab Sahraoui (%)	1 (2.4)	2 (4.7)	1.00
Arab Amazigh (%)	3 (7.1)	1 (2.3)	0.360
Rifi (%)	5 (11.9)	2 (4.7)	0.265
Behaviours			
Tobacco (%)			
Yes	37 (88.1)	7 (16.3)	0.000
No	5 (11.9)	36 (83.7)	0.000
Cannabis use (%)			
Yes	10 (23.8)	6 (14)	0.279
No	32 (76.2)	37 (86)	0.279
Alcohol use (%)			
Yes	13 (31)	0	0.000
No	29 (69)	43 (100)	0.000

Table 2. Clinical Characteristics of the Patients and Histological Type

Clinical characteristics	Case (n= 42)
Symptoms	
Caught	
Yes (%)	32 (76.19)
No (%)	8(19.04)
Unknown (%)	2 (4.76)
Haemoptysis	
Yes (%)	17(40.5)
No (%)	23 (54.76)
Unknown (%)	2 (4.76)
Weight loss	
Yes (%)	23 (54.76)
No (%)	17 (40.48)
Unknown (%)	2 (4.76)
Chest pain	24(57.1)
Yes (%)	16 (38.1)
No (%)	2 (4.76)
Unknown (%)	

Table 2. Continued

Clinical characteristics	Case (n= 42)
Dyspeniea	25 (59.52)
Yes (%)	15(35.71)
No (%)	2 (4.76)
Unknown (%)	
Fever	5 (11.9)
Yes (%)	35 (83.33)
No (%)	2 (4.76)
Unknown (%)	
Histologic type of Lung cancer	
Adenocarcinoma (%)	17 (40.48)
Non-Small cell carcinoma (%)	6 (14.28)
Small cell carcinoma (%)	3 (7.1)
Squamous cell carcinoma (%)	16 (38.1)
Stages of Lung cancer	
Stage III (%)	20(47.62)
Stage IV (%)	20 (47.62)
Unknown (%)	2(4.76)

Table 3. Distribution of *C. pneumoniae* in Lung Cancer Patients and Controls

	Case (n= 42)	Control (n = 43)	P-value
PCR+ (effective) (%)	14 (33.3)	2 (4.7)	0.001

Molecular detection of C. pneumoniae in lung cancer patients and controls

Even though the nested PCR detected *C. pneumoniae* in both patients and controls DNA samples, the statistical analysis revealed that the LC patients were significantly more affected with *C. pneumoniae* than healthy controls ($p < 0.05$) (Table 4).

To compare the relation between *C. pneumoniae* infection, cancer stages, and histological type of LC, The nested PCR results have been stratified in patients according to the diverse cancer sub-types. The nested PCR results confirmed the presence of *C. pneumoniae* infection in cancer studied stages and types, with dominance in patients diagnosed in stage III (35.71%) and in patients with adenocarcinoma subtype (50%) (Table 5).

Assessment of the C. pneumonia role as positivity and risk factors for LC development

To assess the role of *C. pneumonia* in regard to LC development, we performed a multivariate logistic regression analysis; our results revealed that *C. pneumoniae* is a dependent risk factor for LC development as well as Tabaco consumption. In order of importance we cite: *C. pneumoniae* (OR=0.098 CI 95% [0.021–0.463]), and tobacco use (OR= 0.026 CI 95% [0.008–10.09]).

Discussion

Lung cancer is a multifactorial disease. Several investigations confirmed that microbial agents may play an important role in this disease development (Liu et al., 2020). According to the World Health Organization, 18.6 % of deaths in Morocco are due to LC. The aim of this study was to evaluate the association between *C. pneumoniae* and LC in Moroccan patients through a case/control study. In the present study, we selected a molecular methodology, based on nested PCR to detect the *C. pneumoniae* DNA in the Peripheral blood mononuclear cells (PBMCs) of cases and controls.

Table 4. Distribution of *C. pneumoniae* According to Stage and Histological Type of Lung Cancer (n= 42).

Variables	PCR+ (n= 14)	PCR- (n = 28)	P-value
Stages of Lung cancer			
Stage III (%)	5 (35.71)	15 (53.57)	>0.05
Stage IV (%)	7 (50)	13 (46.43)	>0.05
Histologic type of Lung cancer			
ADK (%)	7 (50)	10 (35.71)	>0.05
NSCLC (%)	0	6 (21.43)	>0.05
SCLC (%)	1 (7.14)	2 (7.14)	>0.05
Scc (%)	6 (42.85)	10 (35.71)	>0.05

Abbreviations: ADK, Adenocarcinoma; NSCLC, Non-Small Cell Lung Cancer; SCLC, Small cell lung cancer; Scc, Squamous Cell Carcinoma.

Demographic data analysis shows that the sex ratio between men and women is 41 among LC patients. This masculine predominance is similar to that described in the Moroccan lung cancers and as well in other North African countries lung cancer registers (Rabat cancer registry., 2012; Benarba et al., 2014; Tunisian minister of health., 2015; Casablanca cancer registry., 2016).

A high sex ratio is classically observed in the series where female smoking is less dominant than male smoking (Demirci et al., 2013; Refeno et al., 2015). In our serie, most patients diagnosed with lung cancer were smokers (90.5 %), confirming that smoking habit is the main contributing risk factor of LC.

A multitude of extra risk factors for LC are known. One extra risk issue of potential interest is cannabis, effectively numerous epidemiological studies cover a link between cannabis consumption and LC (Berthiller et al., 2008; Underner et al., 2014; Baumeisteret al., 2021). Indeed, recent meta-analysis study reported a significant association of the cannabis use with a higher risk of developing LC (Ghasemiesfe et al., 2019); this association

Table 5. Evaluation of the Role of *C. pneumoniae* Infection as Risk for LC.

	Case (42)	Control (43)	Univariate analysis				Multivariate analysis			
			P	OR	CI for OR 95%		P	OR	CI for OR 95%	
Gender	Male: 41	Male: 39	0.179	4.205	-0.146	0.771	0.208	4.205	0.650	39.293
	Female: 1	Female: 1								
Age	n= 42	n= 43	0.132	0.942	-0.03	0.004	0.098	0.942	0.878	1.001
Tabaco use	Yes: 37	Yes: 7	0	0.024	-0.875	-0.572	0	0.026	0.008	0.09
	No: 5	No: 35								
Cannabis use	Yes: 10	Yes: 6	0	0.476	-0.882	-0.483	0.250	0.519	0.170	1.586
	No: 32	No: 37								
Alcohol use	Yes: 13	Yes: 0	0.006	0.238	-0.57	-0.101	0.998	0	.	.
	No: 29	No: 43								
C.pneumoniae	Yes: 14	Yes: 2	0.001	0.098	-0.729	-0.209	0.003	0.098	0.021	0.463
	No: 28	No: 41								

P, Level of significance; OR, odds ratio; CI, confidence interval

can be explained by the following arguments:

First, cannabis and tobacco contain comparative groupings of polycyclic aromatic hydrocarbons (PAHs) and other cancer-causing agents (Moir et al., 2008). Second, [Δ9-Tetrahydrocannabinol) THC which is contained in cannabis has immunosuppressive properties and aid in LC growth (Bhattacharyya et al., 2015). Third, immuno-histologic contemplate recognizing atomic deregulation in lung biopsies acquired from cannabis smokers, including overexpression of Ki-67 and Epidermal development factor receptor EGFR (Tashkin and Roth., 2019).

Furthermore, we found that alcohol use was related to a higher LC development ($p<0.05$), another study using a large cohort and limiting investigations to never smokers, shows a slightly association of alcohol consumption with risk of lung malignancy (Freudenheim et al., 2005). In contrast, Gordon Fehringer and his collaborators reported a negative association between overall liquor consumption and lung cancer for low and moderate drinking (Fehringer et al., 2017). The same conclusion was reported by a meta-analysis which included 26,509 cases (Bagnardi et al., 2015).

In our patient population, the symptoms were dominated by cough, Dyspnea, chest pain and weight loss, hemoptysis, and finally fever. Moreover, adenocarcinoma was the most frequent histological type of LC, which is in agreement with the data from the Rabat and Casablanca cancer registers (Rabat cancer registry., 2012; Casablanca cancer registry., 2016).

Several studies had demonstrated the relationship between *C. pneumoniae* and lung carcinoma using *C. pneumoniae* antibodies titers (Anttila et al., 2003 ; Liu et al., 2010); The work of Anttila et al., (2003) on 58 women confirmed histologically with primary LC, showed a prevalence of *C. pneumoniae* IgG of 96.55%, and a prevalence of *C. pneumoniae* IgA of 50%, another work of Liu et., (2010) all among women in china found 62% of positive *C. pneumoniae* IgG, and a correlation of 1.366 between LC and positivity of *C. pneumoniae* IgG. However, to confirm the involvement of *C. pneumoniae* in LC disease, direct methods like culture and molecular detection are the best diagnostic tools. In our investigation, we opted to evaluate the association of *C. pneumoniae* with LC, performing a molecular screening in a case/control study.

Based on multivariate stepwise logistic regression analysis, our results assess that *C. pneumoniae* is an dependent risk factor for LC (OR=0.098 CI 95% [0.021–0.463]), Contrary to our results, R. SESSA et al who investigated *C. pneumoniae* in lung tumor tissue using real-time PCR did not find the involvement of *C. pneumonia* in the pathogenesis of LC (Sessa et al., 2008). Other studies detecting *C. pneumoniae* infection in patients with LC based on serologic criteria showed a higher prevalence of developing LC for subjects who had *C. pneumoniae* IgG+ and *C. pneumoniae* IgA+ wish confirmed the association between *C. pneumoniae* and LC (Koyi et al., 1999 ; Koyi et al., 2001 ; Kocazeybek., 2003 ; Littman et al., 2004 ; Chaturvedi et al., 2010 ; Zhan et al ., 2011 ; Hua-Feng et al., 2015 ; Xu et al., 2020).

Several mechanisms have been proposed to explain how infection with *C. pneumoniae* increased the risk of LC disease. The plausible relationship hypothesis is Likely due through mediators of inflammation. Effectively, epithelial cells recognize Chlamydia l antigens through cell surface receptors, endosomal receptors, and cytosolic innate immune sensors. Activation of these receptors initiates the release of pro-inflammatory cytokines and chemokines, which recruit inflammatory cells (Elwell et al., 2016). Indeed, it was demonstrated that *C. pneumoniae* infection can play a role in the initiation, the progress or the complication of the inflammatory process resulting to diseases development.

It is known that reactive oxygen species (ROS) produced by various biochemical and physiological oxidative process in the body, at high level play a major role in the damage of protein, lipids and DNA. Via mis-repair or incomplete repair, the accumulation of damaged DNA can lead to mutagenesis and cell transformation (Prasad et al., 2017). On the other, side chronic *C. pneumoniae* infection could liberate Chlamydia l heat shock protein-60 (CHSP-60), which may act as a part of the pathogenesis of lung carcinoma (Wang et al., 2019). Furthermore, the relationship between *C. pneumoniae* infection and LC risk could vary when combined with environmental factors. Indeed, among our patients, a significant association was found between *C. pneumoniae* and LC among Tabaco users (OR= 0.026 CI 95% [0.008–10.09]). Several research papers have reported a high prevalence of LC among smoking adults (Le Faou et al., 2005).

In conclusion, *C. pneumoniae* infection is potentially associated with primary LC in the Moroccan population and has combined effects with Tabaco consumption. However, in order to validate the relationship between *C. pneumoniae* and primary lung cancer Future prospective studies with extensive population are required; these studies will allow a better knowledge of the pathogenic role of *C. pneumoniae* infection in Lung malignancy.

Abbreviations

C. pneumoniae: *Chlamydia pneumoniae*; EB: elementary body; HIV: human immunodeficiency virus; IgA: immunoglobulin A; IgG: immunoglobulin G; LC: Lung cancer; PBMCs: Peripheral blood mononuclear cells; PCR: Polymerase Chain Reaction; RB: reticulate frame; TBC: Transfusion Blood Center;

Author Contribution Statement

MC wrote the manuscript analysed and interpreted the results ; KS and FR were responsible for the study design; MM, KH, AZ, NT, IAR, HS, AB, RZ, and JEB provided participants included in this project; MC, HC, and, MF collected the participants samples and datas; FR, KS, and HO provided study materials; MC, HC, MF, and MA carried out the laboratory part of the work; FR , and KS validated the laboratory examination, paper drafting and reviewing; IB validated the statistical analysis.

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Ethics approval

This study is part of an approved student thesis, approved by Ethics Committee for Biomedical Research in the Faculty of Medicine and Pharmacy of Rabat (CERB).

Ethical committee approval

This study is approved by Ethics Committee for Biomedical Research in the Faculty of Medicine and Pharmacy of Rabat (CERB), (IORG Number: IORG0006594) Morocco. The committee's reference number: 38/13.

Availability of data

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

Consent to participate

All the participants were volunteers and provided written, informed consent prior to participation in the study.

Conflict of Interest

Authors declare that they have no conflict of interest.

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