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T H E S I S P R O J E C T

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Baseline factors associated with subsequent kidney involvement in patients with systemic lupus erythematosus. A retrospective cohort study with external validation

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Contents

Abbreviation	Explanation
ACR	American College of Rheumatology
AGR	Albumin-to-globulin ratio
aHR	Adjusted hazard ratio
AIC	Akaike information criterion
AUC	Area under the ROC curve
BILAG	British Isles Lupus Assessment Group
CIF	Cumulative Incidence Function
ESKD	End-stage renal disease
EULAR	European Alliance of Associations for Rheumatology
Harrell's C	Harrell's concordance index
HR	Hazard ratio
ISN/RPS	International Society of Nephrology/Renal Pathologic Society
KM	Kaplan-Meier
LN	Lupus nephritis
LRT	Likelihood ratio test
MLE	Maximum likelihood estimation
NETs	Neutrophil extracellular traps
NP	Neuropsychiatric
ROC	Receiver operating characteristic
SLE	Systemic lupus erythematosus
SLICC	Systemic Lupus International Collaborating Clinics

1. Introduction

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease with protean clinical manifestations and an erratic clinical course. Among its various clinical manifestations, kidney involvement represents a major milestone, with long-standing prognostic implications on morbidity and mortality (Anders *et al.*, 2020). To underline this, the recently updated European Alliance of Associations for Rheumatology (EULAR) recommendations for SLE highlight the need for "regular screening for organ involvement (especially nephritis)" within one of the overarching principles for the management of the disease (Fanouriakis *et al.*, 2023). In this regard, it is essential to identify patients who are at high risk for subsequent development of lupus nephritis (LN) [term used in this document interchangeably with "kidney involvement"], if the latter is not present at disease onset. Various cohort studies have shown that the prevalence of LN within SLE patients is higher in men (versus women), juvenile-onset (vs. adult-onset disease) and certain races or ethnicities, such as non-Whites, compared to Whites (Stefanidou et al., 2011; Hoffman et al., 2009;Maningding et al., 2020).

Kidney involvement often occurs as a presenting manifestation in patients with SLE, with the majority of patients developing LN within the first five years from disease onset. In the 'Attikon' lupus cohort, we found that kidney involvement was evident in a little over 10% of patients at disease onset, reaching a cumulative prevalence of 21% after a median five years of follow-up (D. Nikolopoulos *et al.*, 2020). Among patients initially presenting with mild disease (according to British Isles Lupus Assessment Group (BILAG) index and initial treatments administered), males, those with neuropsychiatric involvement, as well as those positive for anti-ds DNA at baseline were at higher risk to subsequently progress to a more severe disease phenotype during follow-up, including some forms of LN (Di. S. Nikolopoulos *et al.*, 2020).

In the present study, we used data from our cohort to discern predictive factors at disease onset for the development of incident LN, in order to identify patient subgroups in need for a more vigilant monitoring, or earlier use of immunosuppressive drugs and/or biologics, that may decrease this risk. To strengthen our findings, we aimed to cross-validate our analysis in an independent patient cohort (University of Crete Lupus registry) in Greece.

In the following chapters we will summarize current knowledge on SLE and LN pathophysiology (Chapter 2), highlighting specific risk factors for subsequent LN development, according to the

literature. This will serve as the basis for research methodology (Chapter 3), analysis results (Chapter 4) and, finally, discussion (Chapter 5).

2. Theoretical background

This section establishes the theoretical foundation for the subsequent chapters. We will briefly review SLE epidemiology, pathophysiology and main clinical manifestations. We also provide an overview of LN, which was the focus of this research, focusing on the clinical significance of early diagnosis. Next, we explore current literature on mechanisms by which SLE progresses to LN, highlighting known risk factors and knowledge gaps, particularly for the Greek population.

2.1. Systemic lupus erythematosus

SLE is the prototypic systemic autoimmune disease, characterized by multi-systemic manifestations and autoantibodies production. Its erosive skin manifestations were first described during the Middle Ages as a "wolf's bite" (hence the term "lupus") and in 1846 with the "butterfly" metaphor, by Ferdinand von Hebra (**Figure 2-1**), referring to the disease's characteristic malar rash. Lupus' systemic nature was recognized *later* by Moriz Kaposi (1837-1902), Osler in Baltimore and Jadassohn in Vienna.

Figure 2-1. *A woman with the SLE-characteristic malar rash, as depicted in Ferdinand von Hebra 'Atlas of Skin Diseases' (image under public domain).*

2.1.1. Epidemiology

Approximately 400,000 people are newly diagnosed with SLE each year worldwide, according to a 2023 systematic review and modeling study (Tian *et al.*, 2023). Incidence rates vary substantially worldwide, from 1 to 23 per 100,000 persons/year, and are influenced by geographical regions and differences in the distribution of factors including age, race and gender of the population. Global SLE prevalence and affected population are estimated to be 43.7 (95% CI: 15.86-108.92) per 100,000 persons and 3.41 million people, respectively.

Most cases are diagnosed between the age of 15 and 64 years old (Boddaert *et al.*, 2004). Women are affected 8 to 12 times more than men (Alonso *et al.*, 2011). This difference is greater among patients presenting in their twenties and gets alleviated at older age, reaching an equal sex ratio for patients with disease onset after the age of 80 years (Hermansen *et al.*, 2016). There is a trend for higher age at SLE diagnosis among men, as well as higher mortality, compared to women. Race is also strongly associated with SLE, with African-American and Hispanic descendants being affected more often and with more severe manifestations than Caucasians. This may be attributed to genetic as well as socioeconomic factors (Ugarte-Gil *et al.*, 2016).

2.1.2. Aetiology

The pathogenesis of SLE remain incompletely understood and includes a complex interplay between genetic and environmental factors. Previous research has confirmed familial aggregation in SLE (Ulff-Møller *et al.*, 2018), highlighting the genetic susceptibility component, with more than 100 associated genes. However, family clustered SLE accounts for only about 10% of the total SLE cases, with most of SLE patients being sporadic cases (Chen *et al.*, 2008). Epigenetic effects further influence the genetic risk for SLE; the most well understood effect being DNA methylation (Javierre *et al.*, 2010). Candidate environmental factors include ultraviolet light, drugs, cosmetic products, smoking, viruses (especially Epstein-Barr virus) and silica from occupational exposures such as painting and foundry work.

Hormonal factors play a key role in SLE aetiology, as shown by the marked predominance of women in SLE, with the highest prevalence in women of childbearing age, but equal prevalence in men before puberty and after menopause. Observational studies on contraceptives use and hormonal replacement therapy for the risk of SLE development have shown conflicting data, while no association was with pregnancies.

Breakdown of immunological tolerance leads to elevated type I interferon levels and aberrant immune responses against nuclear, such as double-stranded DNA, and other self-antigens (Crow, 2023). An overview of key concepts in SLE immunopathogenesis is presented in **Figure 2-2**. Defective clearance of apoptotic cells and debris accumulation elicit immune triggering and activation of Neutrophils, forming extracellular traps (NETs) (Dieker *et al.*, 2016), nucleic acid recognition receptors in dendritic cells, B and T cells, and macrophages. B cells response drives the production of autoantibodies that constitute a hallmark in SLE pathogenesis.

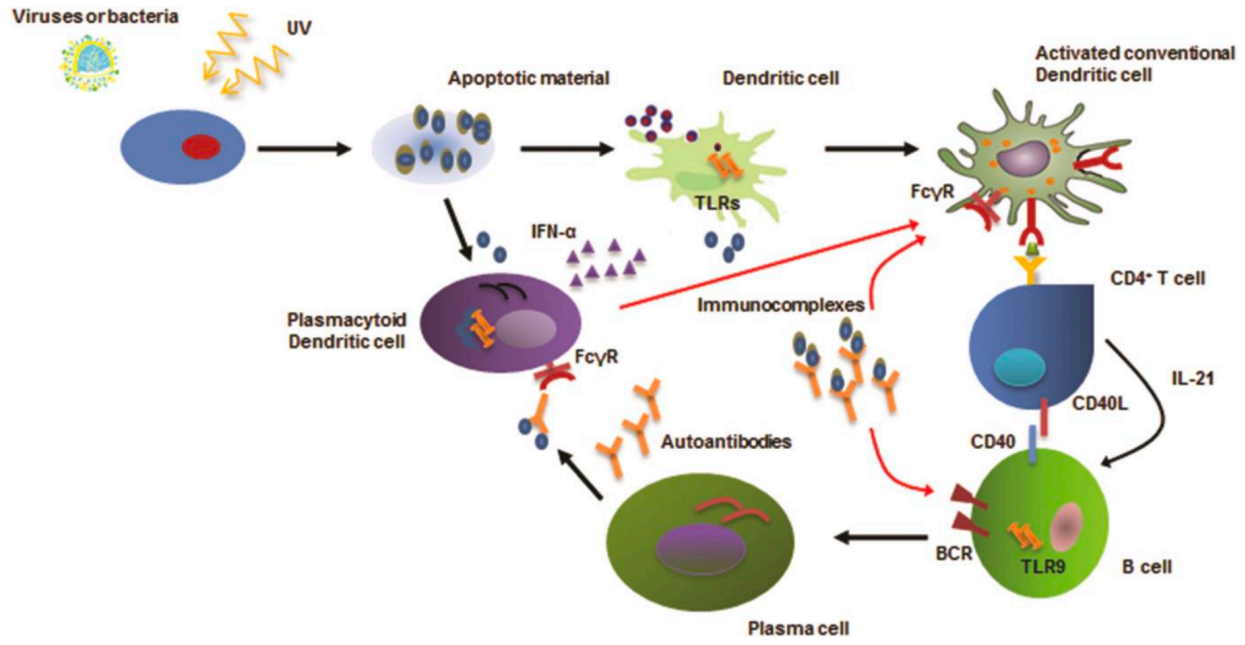

***Figure 2-2**. Overview of key events in immunopathogenesis of systemic lupus erythematosus. BCR, B cell receptor; FcγR, Fcγ receptor; IFN-α, interferon α; TLRs, Toll-like receptors; UV, ultraviolet. (Reproduced with permission from Bertsias et al, Ann Rheum Dis 2010b;69:1603–11.)*

2.1.2. Clinical presentation

SLE can virtually affect any organ and shows great heterogeneity in the incidence and severity of its clinical features. Typical symptoms include fever (>38 °C), hair loss, skin rash, oral ulcers and arthralgias/arthritis (Siegel and Sammaritano, 2024). SLE is a disease that lacks specific pathognomonic features and diagnostic criteria are seldomly used, as clinical diagnosis by an

experience clinician is considered to be the "gold standard". Several classification criteria have been designed, for epidemiological research. Their purpose is to create homogenous cohorts of comparable patients. Until 2019, the 1997 ACR and 2012 SLICC classification criteria were used (**Table 2.2**). The 2019 European Alliance of Associations for Rheumatology (formerly the European League Against Rheumatism)/American College of Rheumatology (EULAR/ACR) (**Supplemental Table S1**) are latest classification criteria, that reach a high sensitivity and specificity of 96.1% and 93.4%, respectively (Aringer *et al.*, 2019). There criteria may also aid in SLE diagnosis, as they identify most patients. Nevertheless, they shouldn't preclude diagnosis or treatment, given the marked variety of the disease's clinical presentation.

Table 2-1. *Revised ACR and SLICC classification criteria for SLE.*

	ACR criteria (1997 update) (Tan et al, 1982; Hochberg, 1997)	**SLICC criteria (2012) (Petri et al, 2012a*)**
Clinical criteria		
Skin	1. Malar rash (fixed erythema, flat or raised, over the malar eminences, tending to spare the nasolabial folds) 2. Discoid rash (erythematous raised patches with adherent keratotic scaling and follicular plugging; atrophic scarring occur in older lesions) 3. Photosensitivity (skin rash as a result of unusual reaction to sunlight, by patient history or physician observation)	1. Acute cutaneous lupus (lupus malar rash, do not count if malar discoid; bullous lupus; toxic epidermal necrolysis variant of SLE; maculopapular lupus rash; photosensitive lupus rash), or subacute cutaneous lupus (non-indurated psoriasiform and/or annular polycyclic lesions that resolve without scarring) 2. Chronic cutaneous lupus (classic discoid rash: localised or generalised; hypertrophic verrucous lupus; lupus panniculitis profundus; mucosal lupus; lupus erythematosus tumidus; chilblain lupus; discoid lupus/lichen planus overlap) 3. Non-scarring alopecia
Ulcers	4. Oral or nasopharyngeal ulceration peripheral joints, characterised by tenderness, swelling or effusion)	4. Oral or nasal ulcers
Synovitis	5. Non-erosive arthritis (involving ≥2 peripheral joints, characterised by tenderness, swelling or effusion)	5. Inflammatory synovitis (in ≥2 joints: (a) Characterised by swelling or effusion, or (b) Tenderness and ≥30 min of morning stiffness)

Serositis	6. Any of: (a) Pleuritis (convincing by a physician or evidence of pleural effusion); (b) Pericarditis (documented by ECG or rub or evidence of pericardial effusion) history of pleuritic pain or rub heard	6. Any of: (a) Typical pleurisy (lasting >1 day, or pericardial pain (pain with recumbency improved by sitting forward, for >1 day), or pericardial effusion, or pericardial rub or pericarditis by electrocardiography pleural effusions, or pleural rub) (b) Typical
Renal disorder	7. Any of: (a) Persistent proteinuria >0.5 g/day, or >3+ if measurement is not performed; (b) Cellular casts: red cell, haemoglobin, granular tubular or mixed	7. Any of: (a) Urine protein/creatinine (or 24 h urine protein) representing ≥500 mg of protein/24 h, or (b) Red blood cell casts
Neurological disorder	8. Any of: (a) Seizures; (b) Psychosis (in the absence of offending drugs or known metabolic derangements)	8. Any of: (a) Seizures; (b) Psychosis; (c) Mononeuritis multiplex; (d) Myelitis; (e) Peripheral or cranial neuropathy; (f) Cerebritis (acute confusional state)
Haematological/ immunological criteria		
Haematological disorder	9. Any of: (a) Haemolytic anaemia (with reticulocytosis); (b) Lymphopenia (<1500/mm3); (c) Thrombocytopenia (<100 000/mm3)	1. Haemolytic anaemia 2. Leukopenia (<4000/mm3), or lymphopenia (<1000/mm3) at least once 3. Thrombocytopenia (<100 000/mm3) at least once
Immunological disorder	10. Any of: (a) Anti-DNA antibody to native DNA in abnormal titre; (b) Anti-Sm (presence of antibody to Sm nuclear antigen); (c) Positive finding of antiphospholipid antibodies (based on: (1) an abnormal serum concentration of IgG or IgM anticardiolipin antibodies; (2) a positive test result for SLE anticoagulant; or (3) a false-positive serological test for syphilis, known to be positive for ≥6 months and confirmed by negative Treponema pallidum immobilisation or fluorescent treponemal antibody absorption test)	4. Anti-dsDNA above laboratory reference range (except ELISA: twice above laboratory reference range) 5. Anti-Sm 6. Antiphospholipid antibody positivity: lupus anticoagulant, false-positive test for syphilis (rapid plasma reagin), anticardiolipin (medium or high titre IgG, IgM, or IgA), or anti-β2 glycoprotein 1 (positive IgG, IgM, IgA) 7. Low complement: low C3, or low C4, or low CH50 8. Direct Coombs test (in the absence of haemolytic anaemia)

Antinuclear antibody	11. Abnormal titre of ANA (by immunofluorescence or an equivalent assay at any time and in the absence of drugs known to be associated with drug-induced lupus)	9. ANA (above laboratory reference range)
Classification of SLE	**At least 4 out of 11 criteria**	**Either biopsy-proven lupus nephritis in the presence of ANA or anti-dsDNA as a 'stand-alone' criterion, or four criteria with at least one of the clinical and one of the immunological/ANA criteria**

ACR, American College of Rheumatology; ANA, antinuclear antibody; SLE, systemic lupus erythematosus; SLICC, Systemic Lupus International Collaborating Clinics; (Tan et al, 1982; Hochberg, 1997); (Petri et al, 2012)

The following sections summarize the most common clinical manifestations. Their frequencies, from an international SLE inception cohort, are presented in **Table 2-2** (Hanly *et al.*, 2007).

Table 2-2. *Frequency of SLE manifestations defined according to ACR classification criteria in the SLICC inception cohort.*

Cumulative ACR manifestations	%
Malar rash	37
Discoid rash	12
Photosensitivity	40
Oral/nasal ulcers	38
Serositis	27
Arthritis	74
Renal disorder	29
Neurologic disorder	5
Haematologic disorder	61
Immunologic disorder	76
Antinuclear antibody	95

ACR, American College of Rheumatology; SLE, systemic lupus erythematosus; SLICC, Systemic Lupus International Collaborating Clinics.

Kidney involvement, the most common major organ manifestation and the focus of this research is more comprehensively reviewed in a separate section (**2.2**).

SLE causes a variety of skin rashes. These include acute, subacute, and chronic forms with multiple clinical and histologic types. The most common, acute cutaneous lupus presents as a widespread or butterfly-shaped facial rash, in up to half of all SLE patients during their illness (Stull, Sprow and Werth, 2023). Subacute lupus shows as a light-sensitive, ring-shaped rash, affecting 10-15% of patients. Discoid lupus, the most prevalent chronic form, features well-defined, scaly red patches that may develop into permanent hair loss. Anti-Ro antibodies are linked to skin involvement.

Musculoskeletal involvement is evident in 53-95% of SLE patients, with joint pain, usually in the hands, with or without inflammation and frequently consists the first clinical manifestation. Radiological damage to joints is uncommon in SLE and suggests another condition like rheumatoid arthritis (Dörner *et al.*, 2022). Jaccoud's arthropathy, a long-term inflammation around joints causing temporary deformity, affects 3-13% of SLE patients.

Haematological abnormalities are common and can be the presenting symptom of SLE patients. Specific criteria for these were defined in the EULAR/ACR classification system. Prior studies show 22%-42% of SLE patients experience leukopenia -primarily lymphopenia- (Carli *et al.*, 2015), thrombocytopenia, or anemia. These manifestations are more frequent in patients with antiphospholipid antibodies (Chock *et al.*, 2019). Macrophage activation syndrome, a life-threatening but rare inflammatory condition, occurs in 0.9-4.6% of SLE cases (Aziz *et al.*, 2021). Neuropsychiatric (NP) manifestations comprise a wide range of central and peripheral neurologic and psychiatric conditions, that do not always imply SLE-related attribution and require thorough evaluation; particularly to exclude infection (Bertsias *et al.*, 2010). A meta-analysis of 22 studies with 6055 patients, reported a pooled prevalece of 52.2% among SLE patients (Meier *et al.*, 2021). Non-specific symptoms, such as headache, mood disorders and cognitive dysfunction accounted for the majority of them. Nevertheless, NP manifestations significantly affect the quality of life and work disability of SLE patients, with unmet needs for better diagnostic and therapeutic tools. Cardiovascular and pleuropulmonary involvement in SLE includes various manifestations. Pericarditis develops in up to a quarter of SLE (Miner and Kim, 2014). A meta-analysis of 39 studies reported pleuritis in 16.5% of patients (Medlin *et al.*, 2018). Rarer complications include valve thickening, regurgitation and vegetations, myocarditis, pulmonary hypertension, interstitial lung disease. Nonbacterial thrombotic endocarditis of the mitral valve, linked to antiphospholipid antibodies, is the most common valvular manifestion but usually follows a benign course.

2.2. Lupus nephritis

2.2.1. Epidemiology

LN is a major milestone in the natural history of SLE. It is a form of glomerulonephritis and represents the most frequent major organ manifestation. Almost half of SLE patients develop LN during the disease trajectory, while 15-20% present with LN at disease onset (Cervera *et al.*, 2003). Prior studies have shown that incidence and prevalence vary depending on the population under study and the criretia used to diagnose SLE and LN, and are overall estimated at 1.3 cases per 100,000 person-years and 21.2 per 100,000 persons, respectively (Hocaoğlu *et al.*, 2023). Kidney involvement appears more frequently among patients with non-European ancestry (African-Americans, Hispanics, Asian ethnicities), compared to European descent patients (Korbet *et al.*, 2007) and in pediatric- versus adult-onset SLE (Amaral *et al.*, 2014). Findings from most cohort

studies suggest a trend for higher prevalence of LN in men compared to women SLE patients, with a male-to-female ratio of 1.1:1 to 1.7:1 (Stefanidou *et al.*, 2011).

2.2.2. Diagnosis

LN typically manifests initially as proteinuria, often without clinical signs of renal disease (also called 'silent' LN), making it a challenging diagnosis, particularly when LN is an inaugural feature of SLE. Newly active LN cases, whether in the context of inception SLE or established disease, may exhibit varied extra-renal SLE manifestations or none at all. Optimal outcomes depend on timely diagnosis and early treatment. Dipstick urinalysis serves as a readily accessible first screening tool, but systematic urinalysis is recommended in all SLE patients.

Diagnosis of LN in SLE patients relies upon i) kidney biopsy, classified according to the International Society of Nephrology/Renal Pathologic Society (ISN/RPS) 2003 classification system (**Table 2-3**), or ii) clinical evidence of persistent proteinuria (>0.5 g per day in a 24-hour urine collection or spot urine protein/creatinine ratio >0.5) and/or evidence of "active" urinary sediment (≥5 red blood cells/high-power field or ≥5 white blood cells/high-power field without infection, or cellular casts of red or white blood cells), according to the 1997 ACR criteria. While classification criteria for SLE rely on clinical findings, the European League Against Rheumatism (EULAR) strongly recommends kidney biopsy for all patients with signs of renal disease, emphasizing early recognition to optimize outcomes.

Significant proteinuria (>0.5 g/day) is nearly ubiquitous in active LN, spanning a spectrum from mild to nephrotic range (>3.5 g/day). "Active" urinary sediment, while less frequently observed, is another important finding in identifying kidney biopsy candidates. Renal biopsy may be warranted in rare instances of SLE patients exhibiting persistent unexplained microscopic hematuria, leukocyturia, or elevated serum creatinine without significant proteinuria. Orthostatic proteinuria may confound urinary protein excretion assessment, therefore the use of first morning urine samples are suggested, for improved reliability. Systematic assessment of laboratory parameters such as anti-dsDNA antibodies, complement levels (C3 and C4), and other biomarkers aids in LN diagnosis and monitoring is mandatory, while monitoring of antiphospholipid antibodies is also suggested, given their potential to induce distinct classes of nephropathies.

***Table 2-3**. The 2003 ISN/RPS classification of LN.*

Class	Definition	Clinical findings	Subclasses
I	Minimal mesangial LN	Normal glomeruli by LM, but mesangial immune deposits by IF	None
II	Mesangial proliferative LN	Purely mesangial hypercellularity of any degree or mesangial matrix expansion by LM, with mesangial immune deposits. A few isolated subepithelial or subendothelial deposits may be visible by IF or EM, but not by LM	None
III	Focal LN	Active or inactive focal, segmental or global endocapillary or extracapillary glomerulonephritis involving <50% of all glomeruli, typically with focal subendothelial immune deposits, with or without mesangial alterations	III (A): active lesions; focal proliferative LN
			III (A/C): active and chronic lesions; focal proliferative and sclerosing LN
			III (C): chronic inactive lesions with glomerular scars; focal sclerosing LN
IV	Diffuse LN	Active or inactive diffuse, segmental or global endocapillary or extracapillary glomerulonephritis involving ≥50% of all glomeruli, typically with diffuse subendothelial immune deposits, with or without mesangial alterations	IV-S[a] (A): active lesions; diffuse segmental proliferative LN
			IV-G[b] (A): active lesions; diffuse global proliferative LN
			IV-S[a] (A/C): active and chronic lesions; diffuse segmental proliferative and sclerosing LN
			IV-G[b] (A/C): active and chronic lesions; diffuse global proliferative and sclerosing LN
			IV-S[a] (C): chronic inactive lesions with scars; diffuse segmental sclerosing LN
			IV-G[b] (C): chronic inactive lesions with scars; diffuse global sclerosing LN
V	Membranous LN	Global or segmental subepithelial immune deposits or their morphological sequelae by LM and by IF or EM, with or without mesangial alterations. May occur in combination with class III or IV, in which case both classes are diagnosed. May show advanced sclerosis	None
VI	Advanced sclerotic LN	≥90% of glomeruli globally sclerosed without residual activity	None

EM, electron microscopy; IF, immunofluorescence; LM, light microscopy; LN, lupus nephritis. [a]Diffuse segmental LN indicates that 50% or more of the involved glomeruli have segmental lesions (that is, glomerular lesions involving less than 50% of the glomerular tuft). [b]Diffuse global LN indicates that 50% or more of the involved glomeruli have global lesions (that is, glomerular lesions involving 50% or more of the glomerular tuft). (Lupus nephritis, Anders et al., 2020, Nature Reviews Disease Primers)

2.2.3. Outcomes

LN significantly impacts the prognosis of SLE patients. Although there is a decreasing rate in recent years, progression to end-stage renal disease (ESKD), the worst important complication of LN, occurs in up to 20% of all patients within the first decade of the disease course (Tektonidou, Dasgupta and Ward, 2016).

Each disease flare results in irreversible nephron loss, that adds up to the natural loss due to physiological ageing (**Figure 2-3**). Impaired renal function at the time of LN diagnosis, as well as poor response to the initial treatment, are associated with poorer long-term outcomes (Mageau *et al.*, 2019). Prognosis is significantly affected by the initial LN classification according to the

International Society of Nephrology/Renal Pathologic Society (ISN/RPS) 2003 classification system and was the main rationale for this classification. Patients with class III, IV or V LN are the highest risk for chronic kidney disease progression, whereas patients on class I or II are at intermediate risk.

Delays in diagnosis or treatment initiation result in greater nephron loss and shorter kidney lifespan, while some patients progress to ESKD after the first first LN episode. Therefore, vigilant surveillance for early signs of kidney involvement and identifying subsets of patients at particularly increased risk for LN development is of utmost imprtance, to improve long-term outcomes of SLE patients.

Figure 2-3. *ESKD in LN patients during their lifetime.*

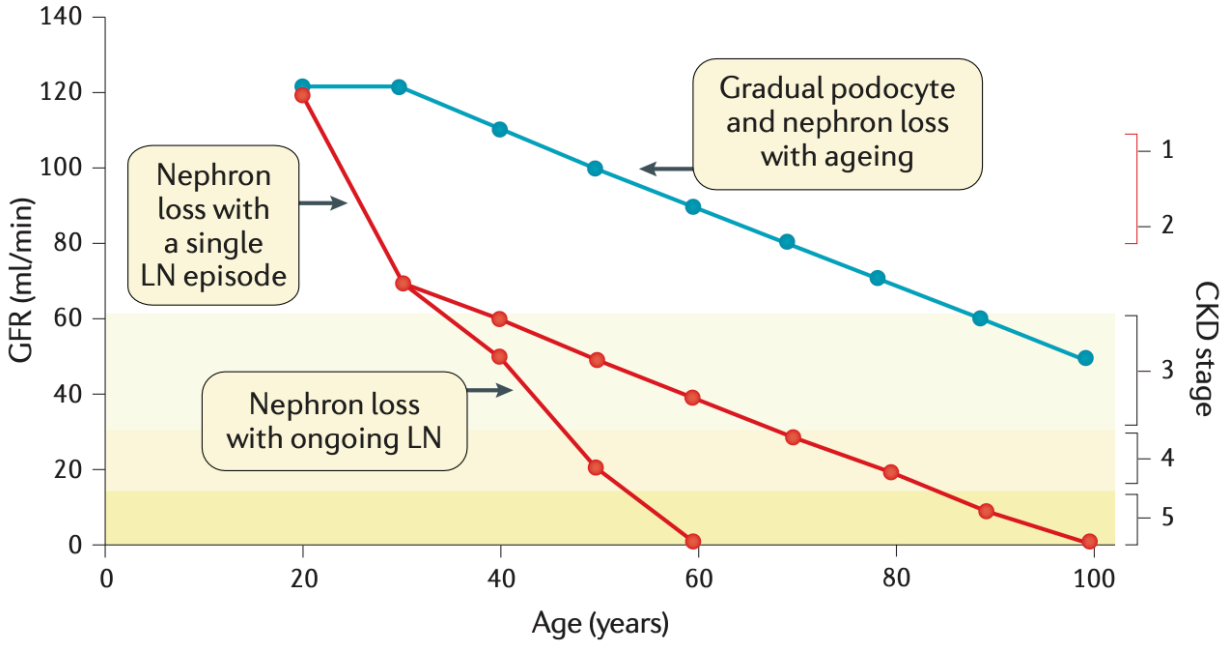

Figure 2-3*. Image reproduced with permission from "Lupus nephritis", Primer Nature, 2020, Anders et al, https://doi.org/10.1038/ s41572-019-0141-9.*

2.2.4. Risk factors

The complete aetiology of SLE and LN remains unknown and includes genetic, hormonal and environmental components. Prior studies have identified several risk factors for SLE, which can be overlapping risk factors for LN.

However, few studies have tried to specifically elucidate kidney involvement factors in SLE patients. A study from 2018 with 278 Korean patients found that higher age, low C3, high anti-dsDNA titre, anti-Sm antibody and low albumin-to-globulin ratio (AGR) were associated with future nephritis, but only anti-Sm and low AGR had a clinically relevant aHR (95% CI) of 2.097 (1.040-4.229) and 4.972 (2.394-10.326), respectively (Kwon *et al.*, 2018). Another Chinese study with 1652 patients, showed that male sex, age <18 years old at SLE diagnosis, with high anti-dsDNA titre were at higher risk for LN, with an adjusted HR (95% CI) of 1.40 (1.12-1.75), 1.50 (1.09-2.06) and 1.57 (1.30-1.90), although the main focus of this study was to construct a machine learning model for future LN prediction (Chan *et al.*, 2023). Additionally, a 2017 study on pregnant women with SLE showed association of LN with low C4 and past kidney disease, but not with anti-dsDNA antibody titre (Buyon *et al.*, 2017).

Despite the considerable volume of research being done on SLE and LN, current literature does clearly elucidate which specific SLE features at diagnosis affect future LN development, whereas some conflicting results have been presented by existing studies. Our research tries to investigate LN risk factors, particularly in the Greek population.

3. Methodology

3.1. Simple analysis

3.1.1. Categorical data

The chi-squared test is a commonly used method to investigate associations between two categorical variables. The null hypothesis, H_0, is that there is no association between them, while the alternative hypothesis, H_0, suggests that there is significant association. The test compares the observed and the expected frequencies in each category, under the assumption of no association. Data are formulated in cross-tabulation tables, with *r* rows and *c* columns. The chi-square statistic is compared to a X^2 distribution with $(r-1)*(c-1)$ degrees of freedom and is calculated as follows:

$$X^2 = \sum_i \sum_j \frac{(O_{ij} - E_{ij})^2}{E_{ij}}$$

$\boldsymbol{O_{ij}}$: the observed frequency in the i_{th} row and j_{th} column

$\boldsymbol{E_{ij}}$: the expected frequency in the i_{th} row and j_{th} column, under the null hypothesis

Another method of analysis for this type of data, when the sample sizes are small, is *Fisher's exact test.*

3.1.2. Continuous data

When dealing with continuous data the variables can take any value within a range. A common way to compare continuous variables is through correlation analysis methods. These include:

- the *Pearson correlation coefficient*, *r*: this test is a measure of the linear association between two continuous variables. It represents the ratio between the covariance of the two variables and the product of their standard deviations, and takes values ranging from -1 to +1. This method assumes normality of the distribution for both variables.

$$r = \frac{\sum(x - \bar{x}) * (y - \bar{y})}{\sqrt{\sum(x - \bar{x})^2 * \sum(y - \bar{y})^2}}$$

x, y: the two variables being investigated

$\bar{x}$, $\bar{y}$: their respective means

- the *Spearman's rank correlation coefficient*: this is the non-parametric counterpart to the Pearson correlation coefficient. It assesses the monotonic relationship of the two variables and also takes values from -1 to +1.

$$r = 1 - \frac{6 \cdot \sum d^2}{n(n^2 - 1)}$$

d: the difference of the order of observation, after observations are placed in ascending/descending order

n: the total number of observations

- *two-sample unpaired t-test*: a statistical test that explores whether there is a significant difference in the mean of a variable between two groups of interest. Its assumptions include normality of the variable's distribution in both groups and equal standard deviation and the statistic has $n_1 + n_2 - 2$ degrees of freedom.

$$t = \frac{x_1 - x_2}{\sqrt{\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}}}$$

x_1, x_2: the two continuous variables

s_1, s_2: standard deviations

n_1, n_2: number of observations for each sample

3.2. Survival analysis

In this chapter we introduce the basic concepts of survival analysis.

3.2.1. Time-to-event data and functions

Survival analysis is the term used when referring to studies where the time from well-defined origin until the event of interest is being analyzed. This kind of analysis is very common in biomedical research and has several advantages over standard methods, such as incidence rate calculations, due to the specific features of survival data; they tend to not be symmetrically distributed, as they are frequently positively skewed (Collet, D., 2008), and are subject to censoring.

Incidence rates are useful when relatively constant risk over time can be assumed, as they provide an average rate during the corresponding period. However, when risk is not constant, the hazard rate and survival analysis provide better estimates. The risk is calculated over short time intervals, during which time is relatively constant. Therefore, the *hazard function,* $h(t)$, is an instantaneous risk of the event happening at time t, given the individual has survived up to time t. It is defined as follows:

$$h(t) = \lim_{\Delta t \to 0} \left(\frac{P(t \leq T < t + \Delta t \mid T \geq t)}{\Delta t} \right)$$

The *survival function,* $S(t)$, which represents the probability of an individual to survive longer than time t, is defined as:

$$S(t) = P(T > t) = exp\{-H(t)\}$$

where $H(t)$ is the *cumulative hazard function*, that is the cumulative risk of the outcome occurring by time t:

$$H(t) = \int_0^t h(u)du$$

3.2.2. Censoring

The survival time of a subject is considered 'censored' when the outcome of interest has not been observed. The most common type is 'right censoring', that can happen when a) the study time has been completed with the patient not experiencing the event (also called administrative censoring), or b) the patient is lost to follow-up. Individuals with censored time contribute some information to the analysis, up to their last follow-up. 'Left censoring' occurs when the actual survival time is less than the one observed. The final type is 'interval censoring', when the survival time is known to lie between two time-points, without exact knowledge of its value. The different types of censoring are displayed in **Figure 3-1**.

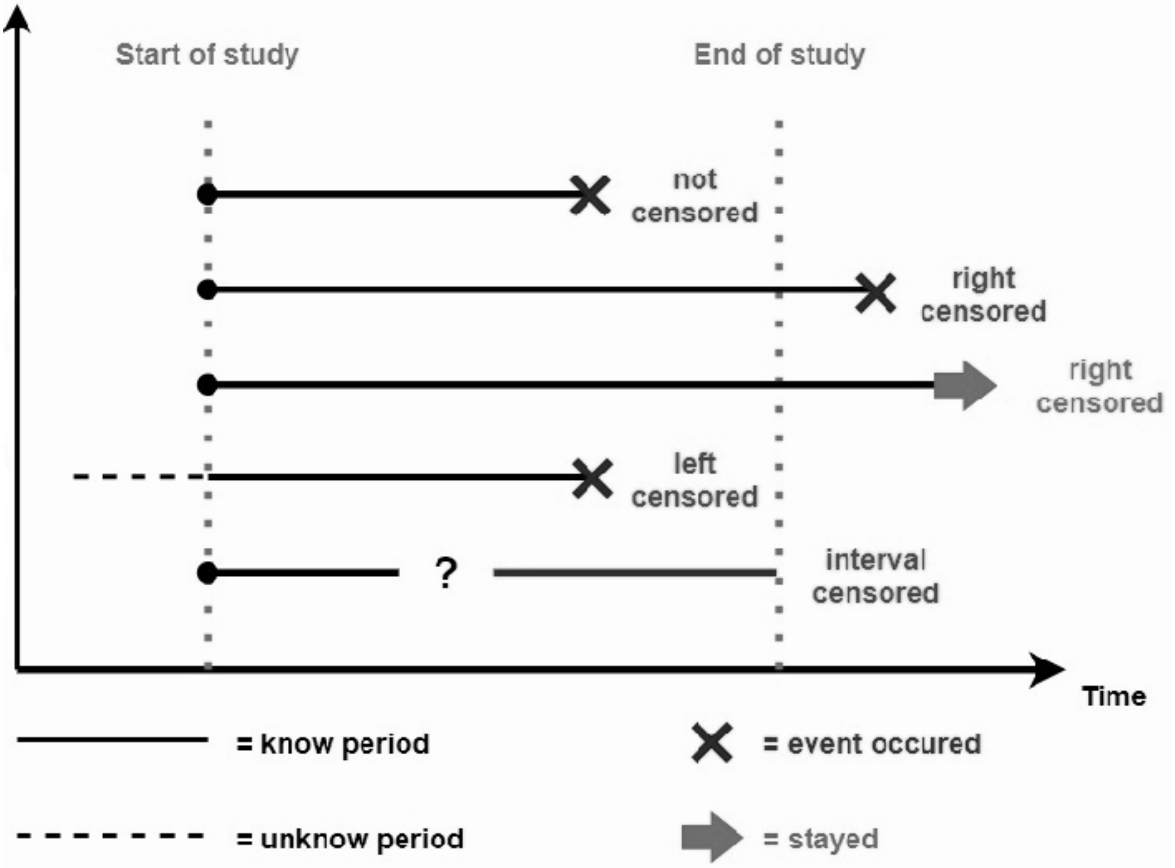

***Figure 3-1**. The different types of censoring in survival analysis (adapted from Pornsawangdee, 2021).*

Standard survival analysis methods assume that censoring is independent of survival time. This assumption is, for example, violated if patients with better prognosis tend to continue participating in the study, while patients with worse prognosis tend to drop out. Another important assumption

is that survival probability does not depend on whether a patient has been recruited early or late in the study. An example of this would be a study where the outcome of a disease gradually got more favorable, as doctors accumulated experience during the study period.

3.2.3. Non-parametric method

The *Kaplan-Meier (KM) estimate*, $\widehat{S(t)}$, is the most frequently used method to estimate the survivor function, when analyzing right-censored data.

$$\widehat{S(t)} = \prod_{j:\tau_j<t} \hat{P}(\tau_j) = \prod_{j:\tau_j<t} \left(1 - \frac{d_j}{r_j}\right)$$

Where $\boldsymbol{\tau_1, \tau_2, \ldots, \tau_k}$ are $\boldsymbol{k}$ distinct and ordered event times observed in a sample of $\boldsymbol{n}$ individuals, $\boldsymbol{t}$ is a time point such that $\boldsymbol{\tau_j < t < \tau_{j+1}}$ and $\boldsymbol{d_t}$ is the number of events at time t.

The KM estimate requires the following conditions:

- The study sample is representative of the population of interest.
- The events happened at the specified times.
- Independent (non-informative) censoring.
- Survival probability independent from recruitment time, relevant to the study duration.

The *log-rank test,* which calculates a chi-squared test based on the differences between the expected and observed number of events, is a method to compare two or more groups of survival times. It does not make assumptions about the distribution of survival time, but is more powerful in the presence of proportional hazards (more on proportional hazards in the following section).

3.2.4. Cox proportional hazards model

The Cox regression model is an extension of the parametric methods discussed previously, that allows for analysis with multiple explanatory variables. A key assumption of this model is the *proportionality of hazards*. The main idea is that the hazard ratio (HR) of the different groups defined by the explanatory variables of the model is constant over time.

Therefore:

$$HR(t) = \psi = constant$$
$$h_i(t) = h_0(t) \cdot \psi(x_i)$$

$\boldsymbol{h_0(t)}$: the baseline hazard fuction of *t*

$\boldsymbol{x_i}$: an observation from a vector x_i of explanatory variables and may include continuous and categorical variables or interaction terms.

$\boldsymbol{\psi(x_i)}$: the HR for subject i, that depends only on the covariates of the model and are *time invariant*

Summarizing, the individual's hazard, $h_i(t)$, is a constant multiple of the underlying baseline hazard, $h_0(t)$, while the constant of proportionality $\psi(x_i)$ is affected only by the values of the individual's explanatory variables.

The full equation of the model becomes the following:

$$\log(hazard\ of\ Y) = \log[h_0(t)] + \beta_1 X_1 + \beta_2 X_2 + \cdots + \beta_p X_p$$

for $\boldsymbol{p}$ covariates.

A key strength of the Cox regression model is that it is possible to make estimations about the β-coefficients without making assumptions about the distribution of the baseline hazard function, $h_0(t)$. Hence it is called a 'semi-parametric' method. The β-coefficients are estimated with the maximum likelihood estimation (MLE) and then the estimates $\hat{\beta}$ are used to estimate the baseline hazard function. This technique is called 'partial likelihood' and relies on the the rankings of the event times, not the actual survival times.

3.2.5. Competing events analysis

In many real-world scenarios patients in a survival study can experience multiple other events, that preclude the observation of the event being investigated. The probabilities of these events are termed competing risks. For example, in a study about death from heart disease, death due to other

causes precludes a person from having the former outcome. Hence, 'cause-specific analysis' has to be utilized.

Standard survival methods and KM estimates under competing risks are not accurate. To illustrate this, if we employ a cause-specific approach, then the KM estimate of the survival probability for the j_{th} cause of event becomes:

$$S_j^{KM}(t) = P(T > t)$$

and all other types of events (competing events) are considered censored. Therefore, we assume that outcome j is the only cause of an event and doesn't consider the other causes. The supplemental probability $F(t) = 1 - S_j^{KM}(t)$ tends to overestimate the cumulative incidence of the outcome j.

An alternative of the KM method is the *Cumulative Incidence Function (CIF).* In this method a new random variable, C, that denotes event type j.

Therefore, the *joint* event probability becomes:

$$F_j(t) = P(T \leq t, C = j), \qquad j = 1, \dots, m$$

and represents the probability that an individual will experience event j by time t.

$$F(t) = \sum_{j=1}^{m} F_j(t)$$

The maximum value of this function is:

$$P(T \leq +\infty, C = j) = P(C = j)$$

and: $\lim_{t \to +\infty} F_j(t) = P(C = j) \neq 1$

This is why $F_j(t)$ cannot be considered a proper probability distribution function and is termed the *subdistribution function*. In the 'cause-specific' context, the survival (also called subsurvivor) function cannot be directly interpreted as a sizable quantity. $F_j(t)$ is often estimated using the *Aalen-Johansen estimator*, which is a generalization of the KM estimator and considers the survival function regarding all types of events (i.e. the disease-free survival).

When multiple covariates are used in time-to-event analysis, adjustments must be made in the standard Cox regression model in order to accommodate for the competing causes of failure. One approach is to use *cause-specific Cox regression*. In this type of analysis, all other event types are considered censored observations. In doing so, the risk set at each event time comprises individuals not having experienced any type of event (i.e. not having failed due to competing events). The results can be interpreted as measures of association between the explanatory variables and the event of interest. However, they cannot be directly interpreted as hazards ratios of standard Cox regression(Andersen and Keiding, 2012).

Fine and Gray (1999) introduced an alternative way to analyze competing risks survival data, by modelling the subdistribution hazard function, $\lambda_j(t)$, which is a direct function of the cause specific CIF:

$$\lambda_j(t) = \lim_{\Delta t \to 0} \frac{P(t < T \leq t + \Delta t, C = j \,|T > t \; or \; (T \leq t \; and \; C \neq j))}{\Delta t}$$

C: the type of event being analyzed

In practical terms, the main difference between the two aforementioned methods lies in the risk set being considered at each event time. In *cause-specific Cox regression*, at every event time the risk set contains individuals that are not censored due to experiencing a competing event, whereas these individuals are retained in the risk set for the *Fine and Gray* model. In that sense, individuals that failed due to a competing event, in the latter model, are considered 'immortal' and the resulting hazard function cannon directly be interpreted as the instantaneous hazard rate at time *t* from cause *j,* given that the individual has not experienced event *j* up to that point.

Both methods complement each other and are frequently used in different scenarios. In aetiological type of research, when the goal is to investigate the factors associated with a particular outcome, the cause-specific Cox model is considered more appropriate (Andersen, P.K. and Keiding, N., 2012). In contrast, when the goal is to make predictions about the incidence of a particular event, for example when estimating a subject's risk of failure over time or when making decisions on population-level interventions, the Fine and Gray model is more appropriate.

3.3. External validation

External validation plays a crucial role in assessing the findings of a study. It allows us to explore whether the findings from examining the study sample are generalizable to a broader population. To this end, the most commonly used techniques used today are a) splitting the starting dataset into two parts, a 'training' and a 'validation' set (this is called internal validation), and b) validating the results in an external set of patients (external validation) (Rahman *et al.*, 2017).

3.3.1. Concordance measures

In survival analysis, a common way to assess a model's predictive performance is by examining its discrimination abilities. This is a measure of how well high and lower risk patients are separated. Subjects at higher risk should tend to exhibit shorter time-to-failure than those at lower risk. To this regard, a key metric used is the concordant pair. It consists of two individuals, among whom the individual with higher predicted risk experiences the event earlier.

Harrell's concordance index estimator (Harrell's C) is frequently used in time-to-event data, where censoring is present. Simply, it expresses the number of concordant pairs, divided by the sum of concordant and discordant pairs.

$$Harrell's\ C = \frac{\#\ concordang\ pairs}{\#concordant\ pairs + \#discordant\ pairs}$$

Or in formula form:

$$C = \frac{\sum_{i,j} I(\tilde{T}_i > \tilde{T}_j) \cdot I(\eta_\xi > \eta_i) \cdot \Delta_j}{\sum_{i,j} I(\tilde{T}_i > \tilde{T}_j) \cdot \Delta_j}$$

$\boldsymbol{i, j}$: the observations pairs

$\boldsymbol{\tilde{T}_i}$: right-censored time-to-event for subject i

$\boldsymbol{\eta_i}$: linear predictor of the hazard function (see section 3.2.4)

$\boldsymbol{\Delta_i}$: indicates whether time $\tilde{T}_i$ has been fully observed ($\Delta_i = 1$), or is censored ($\Delta_i = 0$)

Harrell's C ranges from 0 to 1. Values close to 0.5 indicate no discriminative ability and close to 1 indicate perfect discrimination. Several textbooks suggest that models with C-index close above 0.7 adequately discriminate between risk profiles (Neeman, 2009).

3.3.2. Time-dependent receiver operating characteristics curves

The receiver operating characteristic (ROC) curve assesses the ability of a test (or a predictive model) to discriminate between cases that develop a particular disease and those who do not. They are constructed by plotting the sensitivity (the true positive rate) to 1 minus specificity (the false positive rate), at several classification thresholds. Using this curve, the model's diagnostic accuracy is measured by calculating the area under the ROC curve (AUC),

In the context of time-to-event data the risk of disease is not static, but evolves over time. Consequently, sensitivity and specificity are also time-varying quantities. This led to the adaptation of the AUC concept to survival analysis, through the introduction of time-dependent ROC curves (Bansal and Heagerty, 2019). In this method multiple ROC curves are created, assessing the model's performance, at multiple survival times. This allows for the calculation of different AUC values at each time point and, therefore, AUC becomes a *time-dependent quantity*.

3.4. Patients and data

3.4.1. Patient cohorts

The 'Attikon' lupus cohort is a longitudinal cohort of SLE patients at the 'Attikon' University Hospital, a tertiary centre serving nearly two million residents in Western Attica, Greece. The cohort was established in January 2014 in the hospital's rheumatology department and is still ongoing. It comprises a 'prevalent cohort', which includes all patients diagnosed with SLE, even prior to the establishment of the registry, and an 'inception cohort', which follows patients from diagnosis onwards (D. Nikolopoulos *et al.*, 2020). A comprehensive medical history including demographics, clinical and laboratory information is documented at cohort entry and respective changes are registered at subsequent follow-ups. For the purpose of the present study, we relied on retrospectively collected data for all patients in the registry up to December 2019, for whom the necessary data were available. A comprehensive medical history including demographics, clinical and laboratory information is documented at cohort entry and respective changes are registered at subsequent follow-ups.

The validation cohort was the University of Crete Lupus registry, located at the Department of Rheumatology, University Hospital of Heraklion, which serves as the single referral centre on the island of Crete (population 0.6M), and has a dedicated lupus clinic and registry (Gergianaki et al., 2017; Adamichou et al., 2019).

3.4.2. Ethical Considerations

As the study was based on archival, routinely collected, and de-identified data, no informed consent was required and sought by patients. The study was approved by the 'Attikon' Ethics Committee (protocol number 103/06-03-2014), and, additionally, by the Ethics Committee of the University Hospital of Heraklion (protocol number 5944/14-6-2017).

3.4.3. Patients and definitions

SLE was defined according to the American College of Rheumatology (ACR) 1997 (Hochberg, 1997) and/or the Systemic Lupus Erythematosus International Collaborating Clinics (SLICC) 2012 criteria (Petri *et al.*, 2012), either at the time of diagnosis or cumulatively over the course of the disease. Kidney involvement was defined as:

- i) LN diagnosed by kidney biopsy, classified according to the International Society of Nephrology/Renal Pathologic Society (ISN/RPS) 2003 classification system,
- ii) clinical evidence of LN, according to the 1997 ACR criteria (persistent proteinuria >0.5 g per day in a 24-hour urine collection or a spot urine sample protein/creatinine ratio >0.5, or red blood cell casts).

Of note, for patients who did not undergo kidney biopsy, patient treatment records were reviewed and immunosuppressive therapy for kidney involvement was required to be included in the study, to denote clinically meaningful lupus-related kidney disease and avoid other causes of kidney disease. Survival (LN-free) time was calculated as the interval between SLE and LN diagnosis or last follow-up visit, whichever came first. Since the purpose of this study was to identify associations between clinical and laboratory parameters at SLE onset and subsequent development of LN, patients who manifested LN as a presenting manifestation of their disease were excluded from the analysis.

Baseline data on patients' demographics, including age, sex, disease duration, as well as presenting disease manifestations, complement status and autoantibody profile (coded as binary variables - low C3 and/or low C4 vs both normal, anti-dsDNA (ELISA or CLIFT assay), anti-Sm, anti-Ro, anti-La, anti-RNP, anti-cardiolipin, anti-β2GPI, lupus anticoagulant) use were documented. Of note, laboratory data were not tested in a central laboratory, rather patients were asked to demonstrate their baseline immunologic status and autoantibody profile, which had to be performed in certified laboratories. For patients in the 'prevalent cohort', as part of routine practice in our cohort, detailed information on clinical and laboratory characteristics at the time of disease are specifically sought through patient interview, as well as past medical notes, during the visit of enrolment in the 'Attikon' cohort.

3.4.4. Study outline

For the descriptive analysis, mean and standard deviation or median and interquartile range were used for continuous variables, depending on normality of the distribution of values, and frequency and percentage were used for categorical variables. Differences in baseline data were assessed with t-test for continuous and chi-squared test for categorical variables.

Time-to-event analyses were employed to investigate data behavior over time. The non-parametric Kaplan-Meier (KM) method was used to estimate survival (i.e., LN-free) probability. Survival functions between relevant groups of interest were compared visually utilizing KM curves, and statistically using the log-rank test. Based on the observed data, there was no indication of dependence between the distribution of LN and censoring (which is present when some/partial information about the survival time of a patient is known, but they did not experience the event of interest up to last visit), thus censoring was assumed to be non-informative.

The functional form of 'age' as a continuous variable was investigated with a) categorization into arbitrary groups followed by KM estimation, b) plots of the Martingale residuals against 'age'. The maximally selected rank statistics method, based on log-rank test, was used to determine the optimal cut-off point for age at SLE diagnosis, below which the risk for LN is maximized; this was estimated at 26 years (this is explained in more detail in the 'Results' section). Proportional hazards (PH) assumption for each independent variable was assessed with log-log survival plots, as well as the Grambsch-Therneau test for PH.

Univariate Cox regression was conducted to explore associations of individual baseline variables with the subsequent development of LN. Subsequently, a multivariate model encompassing all statistically significant variables ($p < 0.2$) identified in the univariate step was used. Model selection was based on the likelihood ratio test (LRT) and the Akaike information criterion (AIC). No evidence of interactions between independent variables was detected. Scaled Schoenfeld residuals were used for the evaluation of model fit. Based on the negligible proportion of missing data for crucial variables, no imputation techniques were employed.

Performance of our final multivariate model was evaluated through independent validation, with a focus on measures of discrimination. To this end, KM curves and hazard ratios derived from Cox regression were compared across the two cohorts. Harrell's concordance index (C-index), and time-dependent area under the ROC curve (AUC) were explored (described earlier in 'methodology'), to assess the discriminatory power of the model, between high- and low-LN risk patients, after applying it to the independent validation cohort.

Finally, we explored the consistency of the associations for proliferative (ISN/RPS 2003 class III, IV or mixed) versus non-proliferative LN, for patients with histological diagnosis. The cause-specific Cox model was selected (as opposed to the Fine and Gray model), based on the aetiological focus of this study, with the aim to find associations between baseline factors and

eventual LN development, instead of making accurate predictions on patient survival rates(Lau, Cole and Gange, 2009). The non-parametric Aalen-Johansen method was employed to estimate cumulative incidence curves for this part of the analysis. All analyses were performed using R Statistical Software (v4.3.2; R Foundation for Statistical Computing, Vienna, Austria) and the 'survminer' (v0.4.9; Kassambara, Kosinski and Biecek 2021) and 'cmprsk' (v2.2.11; Gray 2022) R packages.

4. Results

4.1. Patient characteristics

A total of 570 SLE patients from 'Attikon' cohort were evaluated. Overall, 125 patients (22%) developed kidney involvement; 59 (47%) already had evident nephritis at initial presentation and were excluded from the study, as previously explained. Thus, the final sample comprised of 511 SLE patients and 66 cases of 'incident' LN.

The median (IQR) time to development of kidney involvement was 45 (22 – 94) months; of note, 21 patients (32% of all LN cases) developed LN after more than 5 years following disease onset. Non-LN patients had a median (IQR) follow-up time of 23 (11 – 106) months. Total cumulative LN incidence at 1, 5 and 10 years after SLE diagnosis was 12.1%, 15.4% and 17%, respectively. The corresponding cumulative incidence for the study population (i.e. patients with no kidney involvement at baseline) was 2%, 5.7% and 7.4%.

Clinical and laboratory characteristics at disease onset, and comparisons between LN and non-LN groups are shown in **Table 4.1**. Clinical SLE manifestations were similarly distributed among the two groups, with no major differences. On the contrary, patients who developed LN were significantly younger at SLE onset [mean (SD) 29 (13) years vs 40 (15) years, $p < 0.001$] and had a higher frequency of positive anti-dsDNA antibodies [48 (77%) vs 135 (33%), $p < 0.001$], anti-RNP antibodies [10 (20%) vs 33 (9.9%), $p < 0.05$] and low C3 and/or C4 [51 (88%) vs 173 (47%), $p < 0.001$]. Although a tendency for a higher percentage of males was noted in the LN group, this difference was not statistically significant (17% vs 10%, $p = 0.08$).

Table 4.1. Baseline characteristics between patients who subsequently developed lupus nephritis and those who did not.

Characteristic	**Overall** (n = 511)	**Non-LN** (n= 445)	**LN** (n = 66)	**p-value**[a]
Age of SLE Dx, Mean (SD)	38 (15)	40 (15)	27 (13)	**<0.001***
Age of LN Dx, Median (IQR)	28 (21 – 41)	NA	28 (20 – 41)	
Female sex, n (%)	457 (89)	402 (90)	55 (83)	0.08
Fever, n (%)	129 (26)	108 (24)	21 (34)	0.09
Hemolytic anemia, n (%)	16 (3.1)	13 (2.9)	3 (4.6)	0.44
Leukopenia, n (%)	119 (23)	106 (24)	13 (20)	0.46
Thrombocytopenia, n (%)	58 (11)	50 (11)	8 (12)	0.83
Neurologic, n (%)	39 (7.6)	34 (7.6)	5 (7.6)	0.99
Malar rash, n (%)	206 (40)	178 (40)	28 (42)	0.71
Alopecia, n (%)	119 (23)	108 (24)	11 (17)	0.17
Ulcers, n (%)	93 (18)	86 (19)	7 (11)	0.087
Acute CLE, n (%)	337 (66)	297 (67)	40 (61)	0.33
Chronic CLE, n (%)	51 (10)	44 (9.9)	7 (11)	0.83
Serositis, n (%)	60 (12)	52 (12)	8 (12)	0.92

Characteristic	**Overall** (n = 511)	**Non-LN** (n= 445)	**LN** (n = 66)	**p-value**[a]
Arthritis, n (%)	377 (74)	327 (73)	50 (76)	0.70
Anti-dsDNA, n (%)	186 (40)	135 (33)	51 (81)	**<0.001**
Low C3 and/or C4, n (%)	226 (52)	173 (47)	53 (90)	**<0.001**
Anti-Sm, n (%)	34 (8.8)	28 (8.3)	6 (12)	0.42
Anti-Ro, n (%)	126 (32)	106 (30)	20 (39)	0.20
Anti-La, n (%)	50 (13)	42 (12)	8 (16)	0.47
Anti-RNP, n (%)	43 (11)	33 (9.9)	10 (20)	**0.03**
Anti-cardiolipin, n (%)	110 (40)	92 (39)	18 (41)	0.86
Anti-B2GPI, n (%)	66 (26)	56 (26)	10 (24)	0.84
Lupus anticoagulant, n (%)	37 (17)	29 (16)	8 (23)	0.33

[a]: Chi-squared unless otherwise denoted, *: unpaired samples t-test

4.2. Univariate analysis of baseline factors

Comparative analysis was conducted within distinct groups of the study population according to demographic, clinical and laboratory variables, at their baseline values. KM-estimated cumulative incidence curves are presented below, and group differences are assessed with the log-rank test, as well as Cox PH models.

4.2.1. Effect of age and linearity assessment

The use of continuous variables in statistical models presents the challenge of assessing their linearity, to accurately interpret their effect. During exploratory analysis 'age' was initially categorized in 3 groups and the respective KM estimation is illustrated in **Figure 4.2.1**.

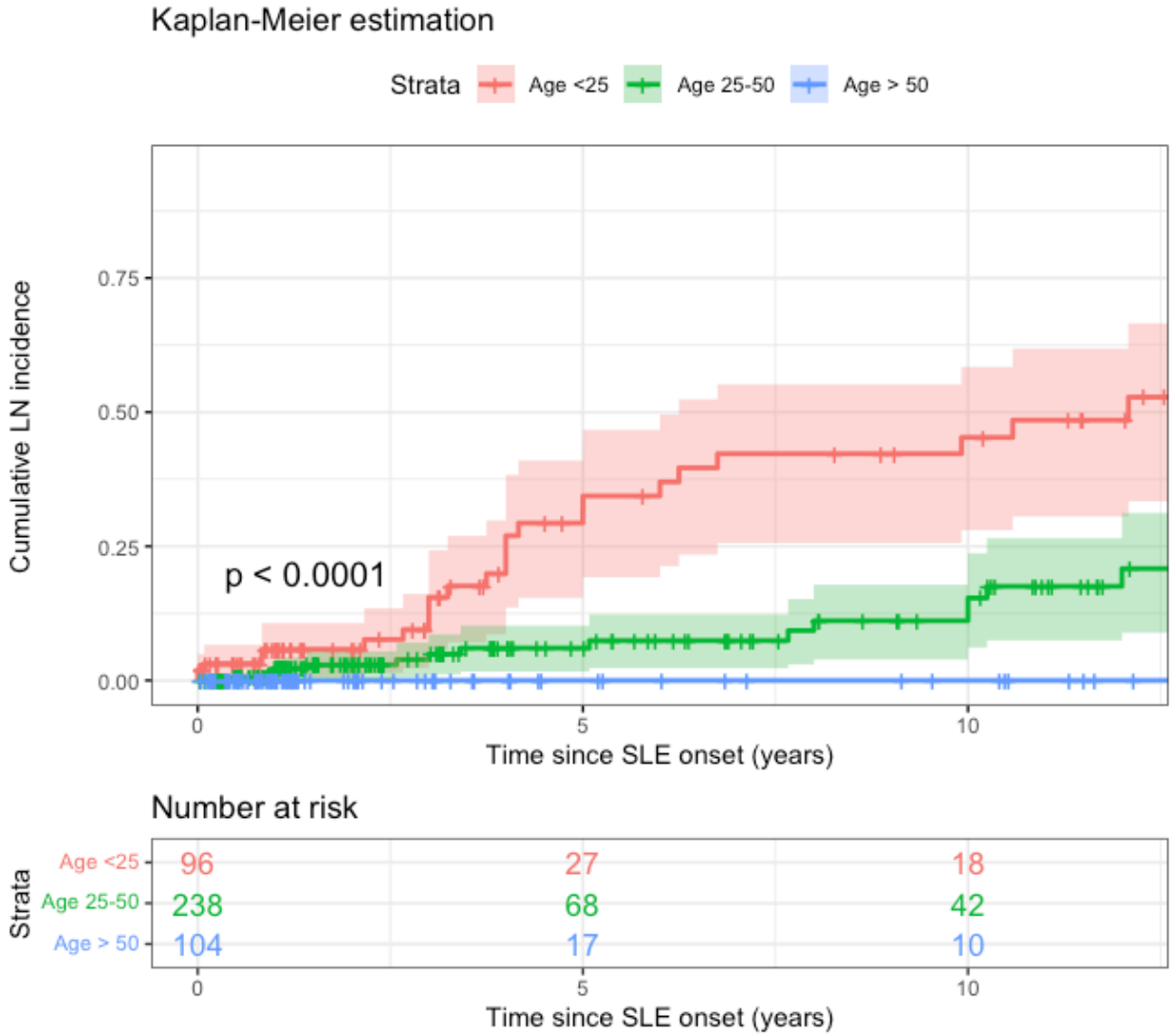

Figure 4.2.1*: Cumulative LN incidence curves according to age at SLE diagnosis; p: p-value of the respective log-rank test.; shaded regions represent the corresponding 95% confidence intervals.*

The youngest group, aged <25 years old, appears to have the highest risk for LN during the whole study duration, while the curves do not intersect at any point. Progressively older age groups seem to have better prognosis. This was also confirmed by the log-rank test, that yielded a p-value < 0.0001, indicating a highly significant association. Therefore, our data suggest a progressively

higher risk for LN for younger ages. However, the *functional form* of 'age' cannot be adequately identified by the categorization of the variable.

Another approach frequently used when assessing linearity, as suggested by (Thernau-Grambch 1990), is to plot the Martingale residuals of the corresponding Cox model to the variable being investigated. This is depicted for 'age' in **Figure 4.2.2**.

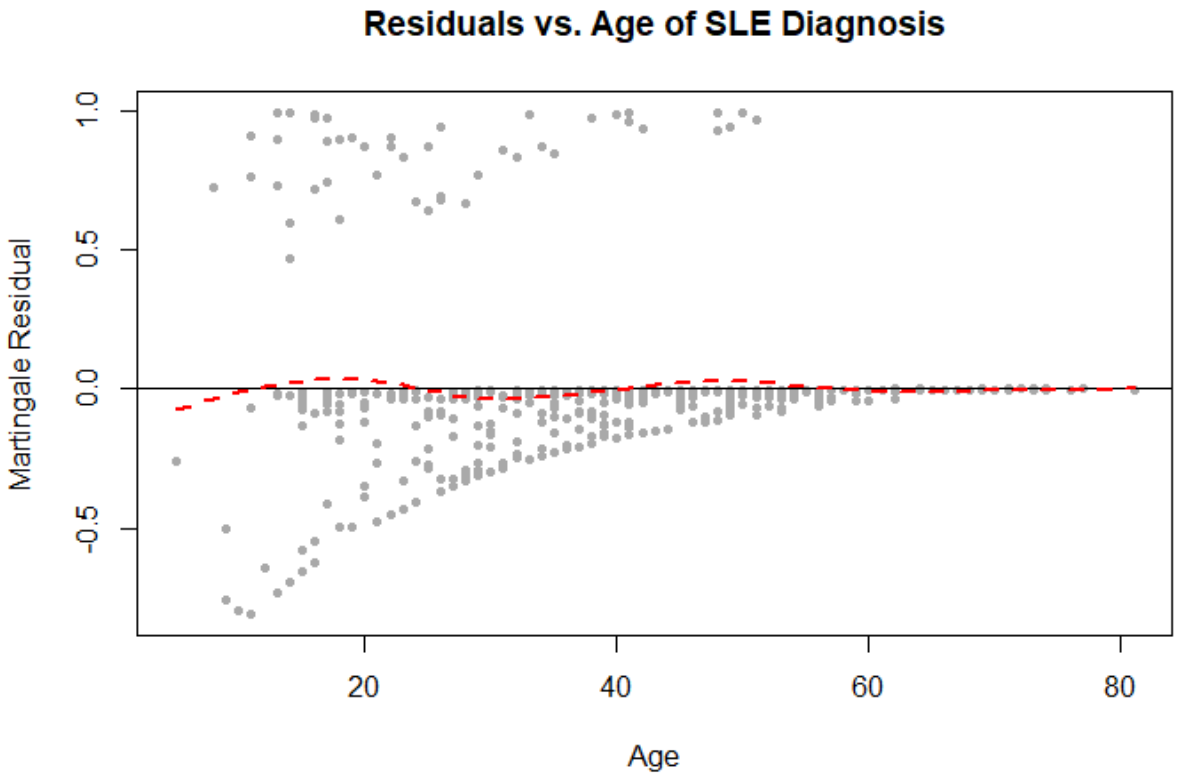

***Figure 4.2.2**. Martingale residuals plotted against 'age' as a continuous variable, to assess its functional form. No indication of non-linearity is detected, shown by the almost horizontal smoothed line.*

As suggested by the above plot, it is safe to assume that age can be used in its continuous form, shown from the almost horizontal red line, representing the Martingale residuals over 'age'. This univariate Cox model revealed 5% lesser risk for LN per greater year at SLE diagnosis (Table 4.2.1).

Characteristic	HR[1]	95% CI[1]	p-value
Age of_SLE_diagnosis (per year)	0.95	0.92, 0.97	**<0.001**

[1] HR = Hazard Ratio, CI = Confidence Interval

Table 4.2.1. *Univariate Cox PH model with age as a continuous variable for the risk of LN.*

Moreover, because of no deviation from linearity, categorization of the continuous variable can be safely conducted. Hothorn and Lausen (2002) suggested a method of determining the optimal cut point of a predictor variable, based on the value that yields the maximum value of the log-rank test, while providing a reasonable sample size in the resulting groups. The distribution of this test is shown in **Figure 4.2.3**, and the optimal cutoff for age was found at 26 years. While using the categorical form has the drawback of loss of some information about the effect of the covariate, the definition of a *pure threshold* has the benefit of improved interpretability, as it gives a clear message to the reader. *Compared to interpreting the coefficient of a continuous variable, providing age stratification gives more actionable information to clinicians*.

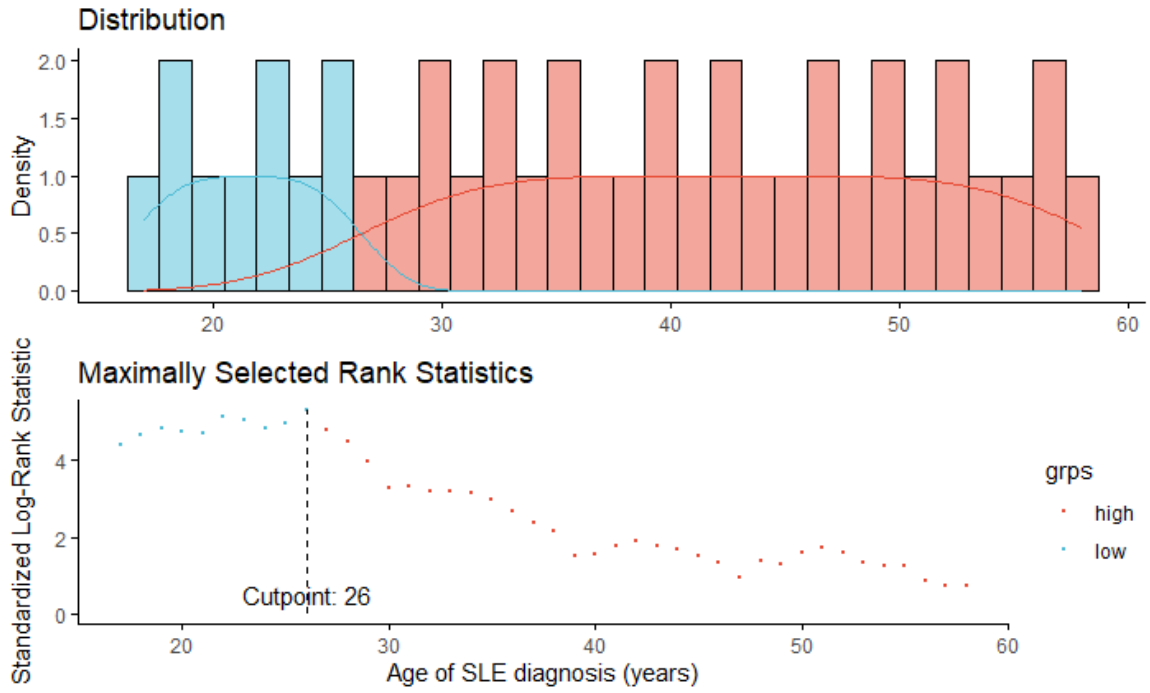

Figure 4.2.3. Age at SLE diagnosis associated with the maximum risk for subsequent kidney involvement**.** Maximally selected ranked statistics analysis, to pinpoint the threshold associated with the maximum risk for subsequent kidney involvement; x-axis represents age at SLE diagnosis as a continuous variable and y-axis depicts the absolute standardized log-rank value. The vertical line indicates the optimal cutoff point, estimated at 26 years.

This method resulted in a HR for LN of 4.10 (95% CI: 2.25-7.47) for patients under 26 years old at SLE diagnosis (**Table 4.2.2**). The corresponding KM estimation is shown in **Figure 4.2.4**.

Characteristic	HR[1]	95% CI[1]	p-value
Age (years) as categorical			
≥26	—	—	
<26	4.10	2.25, 7.47	**<0.001**
[1] HR = Hazard Ratio, CI = Confidence Interval			

Table 4.2.2. *Univariate Cox PH model with age as a categorical variable for the outcome of LN.*

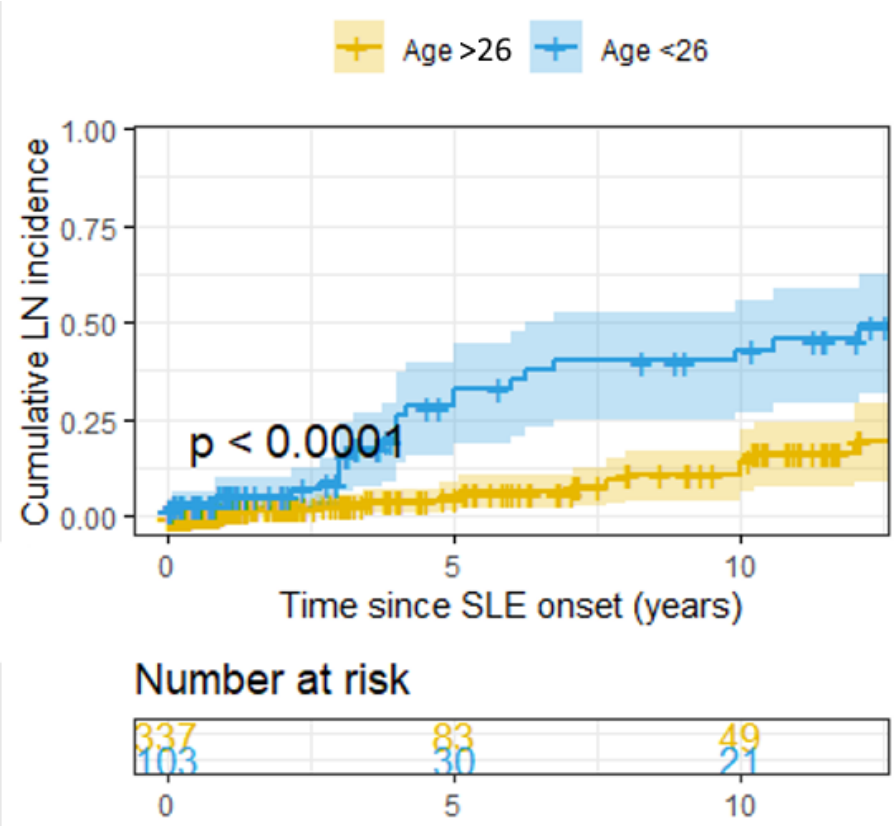

Figure 4.2.4*: Cumulative LN incidence curves according to age category at SLE diagnosis; p: p-value of the respective log-rank test.; shaded regions represent the corresponding 95% confidence intervals.*

4.2.2. Effect of baseline serology

Auto-antibodies (and especially anti-dsDNA) and hypocomplementemia are strongly associated with SLE disease activity and LN flares. Here we explore the baseline anti-dsDNA and complements status in relation to subsequent LN.

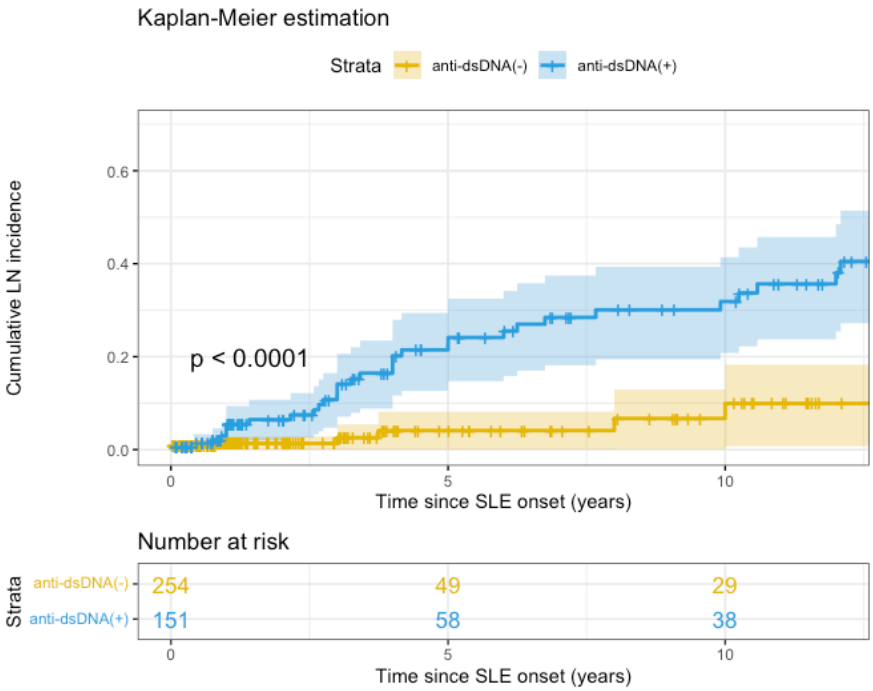

Figure 4.2.5*: Cumulative LN incidence curves according to anti-dsDNA antibody status at SLE diagnosis; p: p-value of the respective log-rank test.; shaded regions represent the corresponding 95% confidence intervals.*

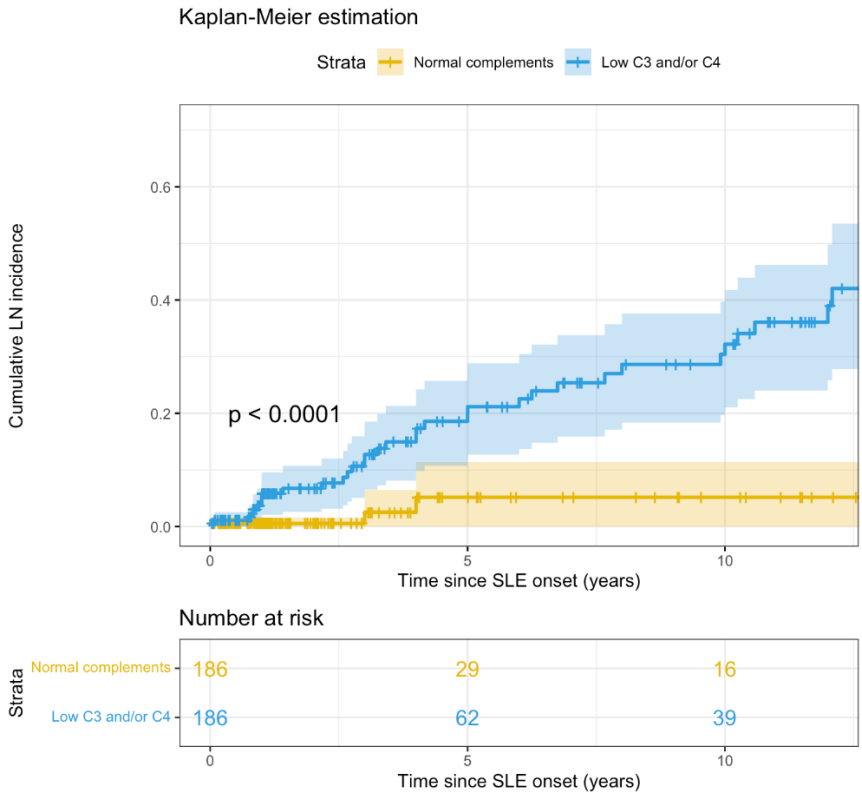

Figure 4.2.6*: Cumulative LN incidence curves according to complements status at SLE diagnosis; p: p-value of the respective log-rank test; shaded regions represent the corresponding 95% confidence intervals.*

In **Figures 4.2.5** and **4.2.6** the KM curves show that patients who are i) anti-dsDNA positive and ii) have low complements, respectively, at SLE diagnosis have a consistently greater risk for subsequent LN. The incidence curves diverge from early in the study and keep separating further over time, without intersecting. The corresponding log-rank tests are also highly significant (p <0.0001 for both variables. Univariate Cox models were fitted (**Tables 4.2.3** and **4.2.4**) with a HR of 5.12 (95% CI 2.39-10.98) and 4.88 (95% CI 1.92-12.43) for anti-dsDNA and low complements, respectively.

Characteristic	**HR**[1]	**95% CI**[1]	**p-value**
anti-dsDNA			
No	—	—	
Yes	4.58	2.12, 9.90	**<0.001**

[1] HR = Hazard Ratio, CI = Confidence Interval

Table 4.2.3. *Cox PH model for baseline high anti-dsDNA antibodies and the outcome of LN.*

Characteristic	HR[1]	95% CI[1]	p-value
Low complements			
No	—	—	
Yes	4.36	1.70, 11.2	**0.002**

[1] HR = Hazard Ratio, CI = Confidence Interval

Table 4.2.4. *Cox PH model for baseline low complements and the outcome of LN.*

We also stratified our study population as per baseline status of both anti-dsDNA and complement status (i.e., single positivity of either, or combined low C3/C4 and positive anti-dsDNA). Notably, combined hypocomplementemia and anti-ds DNA positivity at disease initiation, as depicted in **Figure 4.2.7** suggested there may be an interaction between the two variables. To explore the presence of an interaction multivariate Cox PH models with:

1) anti-dsDNA and 'low complements'
2) anti-dsDNA, 'low complements' and their interaction (which was not statistically significant, p = 0.3)

were fitted (**Table 4.2.5**) and compared by the likelihood ratio test, which was not significant (p-value = 0.31), and the Akaike Information criterion (AIC). AIC value was lower for the model without interaction (350.6 vs 352.3). Both tests showed no evidence of a significant interaction between anti-dsDNA positivity and low complements at baseline, therefore the parsimonious model with no interaction was considered more appropriate.

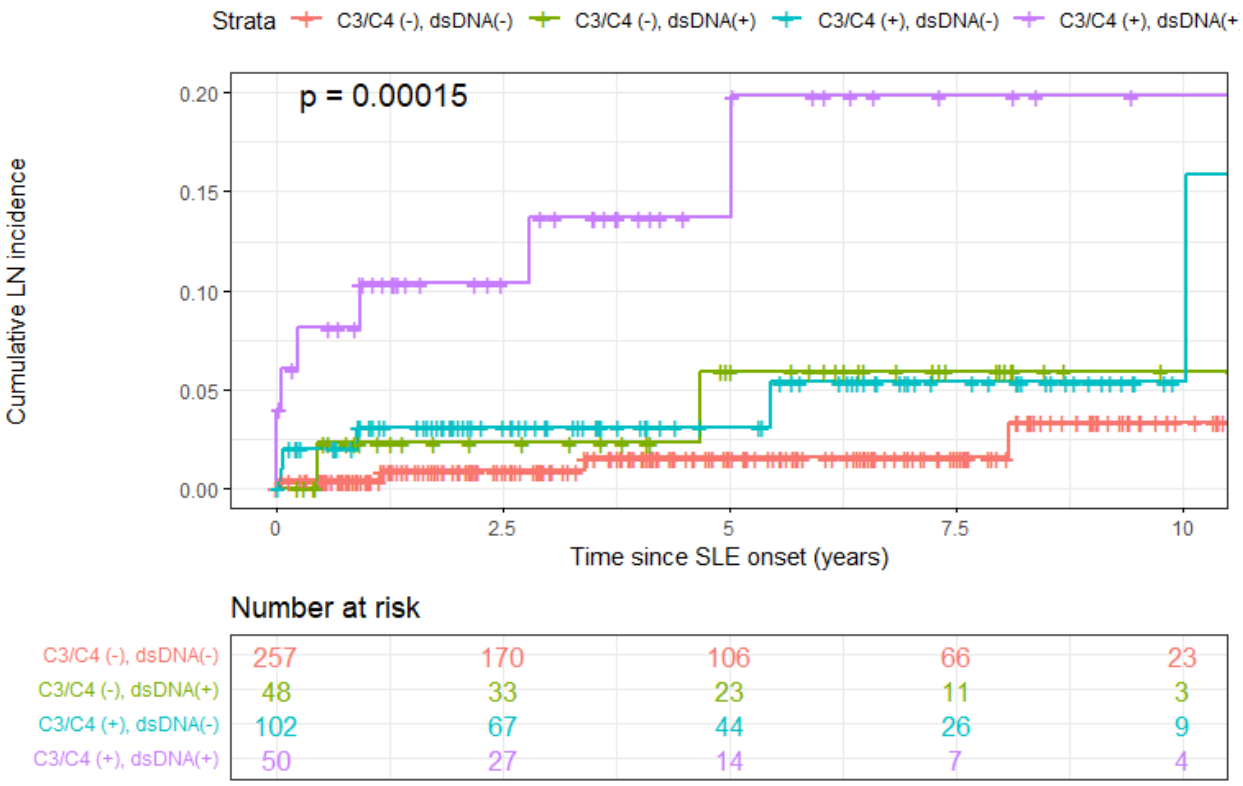

Figure 4.2.7: Cumulative LN incidence curves estimated by the KM method, comparing distinct groups within the study population, based on all possible combinations of baseline anti-dsDNA antibodies; p: p-value of the respective log-rank test.

Cox PH regression	With interaction		No interaction	
Characteristic	**HR**[1]	**p-value**	**HR**[1]	**p-value**
Anti-dsDNA				
No	—		—	
Yes	1.56	0.6	3.45	**0.004**
Low complements				
No	—		—	
Yes	1.71	0.5	3.16	**0.019**
Anti-dsDNA * Low complements			—	
Yes * Yes	2.84	0.3	—	
[1] HR = Hazard Ratio				

Table 4.2.5. *Cox PH regression models with and without interaction between serologic variables for the outcome of LN.*

4.2.3. Effect of gender

The KM plot (**Figure 4.2.8**) shows a higher incidence curve for males. Although the confidence intervals seem to overlap, the curves diverge further as more events are observed over time, along with a statistically significant log-rank test (p-value < 0.05). The corresponding HR from Cox regression was 2.48, (95% CI 1.16-5.30), shown in **Table 4.2.6**, indicating that male patients had a significantly greater risk for subsequent LN compared to females.

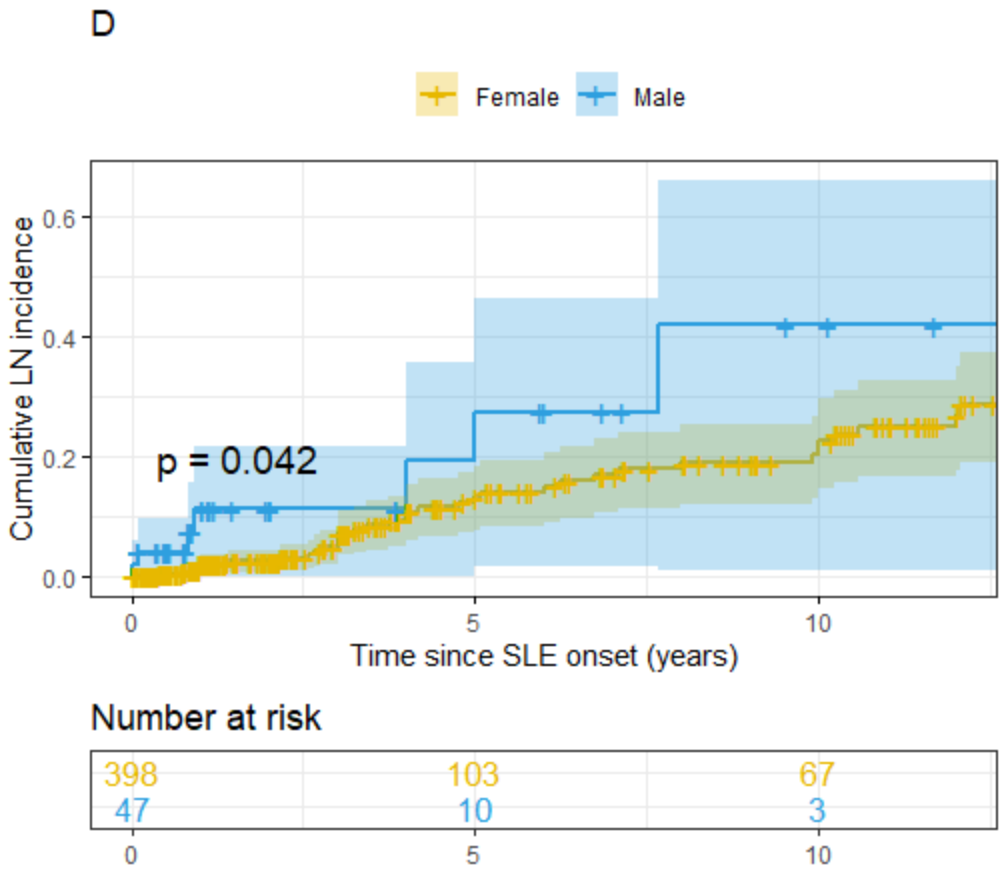

Figure 4.2.8: Cumulative LN incidence curves according to gender; p: p-value of the respective log-rank test; shaded regions represent the corresponding 95% confidence intervals.

Characteristic	**HR**[1]	**95% CI**[1]	**p-value**
Gender			
Female	—	—	
Male	2.75	1.28, 5.92	**0.009**
[1] HR = Hazard Ratio, CI = Confidence Interval			

Table 4.2.6. Cox PH regression for the effect of gender on LN incidence.

Other variables that were tested, but did not reach statistical significance included anti-ENA antibodies (anti-Ro, anti-La, anti-Sm, anti-RNP), antiphospholipid antibodies, as well as all clinical manifestations at disease onset.

4.3. Multivariate analysis

4.3.1. Variable selection

Building upon our findings on univariate analysis, multivariate Cox PH models were constructed, utilizing baseline variables significantly associated with eventual LN development on SLE patients. Variable selection is a crucial part of every analysis, particularly when associations between the outcome and all possible aetiological factors is the main interest of a study. Heinze, et al. (2018), have suggested pragmatic guidelines regarding the choice of variables included in models with censored data, in real-life scenarios. They have used the *term 'events per variable'* (EPV) as a guide that helps identify appropriate procedures such as backwards/forward selection and shrinkage methods. However, 'strong' variables -known from existing literature to have an effect on the outcome- should be retained in the model, regardless of EPV (Heinze, Wallisch and Dunkler, 2018).

Our study sample comprises a total of 66 LN cases and the analysis so far has identified 4 baseline factors associated with the outcome: *age, gender, anti-dsDNA and complements status*. Prior studies, presented in the introductory section 2.2.4, provide some evidence for each one of these covariates, albeit with some conflicting results and in different populations. Therefore, due to the low EPV value of 16.5, and limited number of candidate explanatory variables, all 4 factors were incorporated in the final model.

4.3.2. Multivariate model with age as a continuous variable

Firstly, a Cox PH model with the aforementioned covariates and age of SLE diagnosis as a continuous variable was constructed. Our data suggest that male sex (aHR 3.47, 95% CI: 1.1.49-8.08, $p < 0.01$), age (aHR per year 0.95, 95% CI: 0.92-0.98, $p < 0.01$) and high anti-dsDNA titre (aHR 2.52, 95% CI: 1.04-6.10, $p < 0.05$) were independently associated with LN. Hypocomplementemia did not retain a significant effect, although a clear trend for higher LN risk was evident (aHR 2.21, 95% CI: 0.81-5.98, $p = 0.12$) (**Table 4.3.1**).

Characteristic	HR[1]	95% CI[1]	p-value
Sex			
Female	—	—	
Male	3.47	1.49, 8.08	**0.004**
Age of SLE Dx (per year)	0.95	0.92, 0.98	**0.003**
Anti-dsDNA			
No	—	—	
Yes	2.52	1.04, 6.10	**0.040**
Low C3 and/or C4			
No	—	—	
Yes	2.21	0.81, 5.98	0.12

[1] HR = Hazard Ratio, CI = Confidence Interval

Table 4.3.1. *Cox PH multivariate regression with age as continuous for the outcome of LN.*

4.3.2. Model with age as a categorical variable

The functional form of 'age' as a continuous variable was investigated during univariate analysis (section 4.2.1). This analysis revealed the optimal cutoff was found at 26 years. Subsequently, a multivariate Cox PH model was fitted, treating age as a categorical variable (<26 years vs. ≥26 years). Our data suggest that age of SLE diagnosis below 26 years (aHR 3.71, 95% CI: 1.84-7.48, $p < 0.001$), male sex (aHR 4.31, 95% CI: 1.82-10.20, $p < 0.001$) and high anti-dsDNA titre (aHR 2.48, 95% CI: 1.03-5.97, $p < 0.05$) were independently associated with LN. Conversely, hypocomplementemia did not retain a significant effect, although, again, a clear trend was evident (aHR 2.24, 95% CI: 0.83-6.05, $p = 0.11$) (**Table 4.3.2**). The results are presented in the forest plot of **Figure 4.3.2**.

In line with earlier discussion, the use of categorical variables translates more easily into actionable information for clinicians. Furthermore, between the two presented multivariate models, the latter

(with age as a categorical variable) had a *lower AIC value* (350.6 vs. 355.1). Consequently, this model was selected for the final presentation. The model's full hazard function is presented below:

$$\log(HR) = \log[h_0(t)] + \beta_1 \cdot I(male\ sex) + \beta_2 \cdot I[age(< 26)] + \beta_3 \cdot I(antidsDNA\ positive) + \beta_4 \cdot I(low\ C3/C4)$$

HR: the Hazard ratio for LN

$h_0(t)$: baseline hazard as a function of time

t: time in years

$(male\ sex)$: pseudo-variable representing male sex (female = 0, male =1)

$[age(< 26)]$: pseudo-variable representing age lower than 26 years

$(antidsDNA\ positive)$: pseudo-variable representing anti-dsDNA positivity

$(low\ C3/C4)$: pseudo-variable representing low C3 and/or C4

β_1, β_2 ...: the model's coefficients

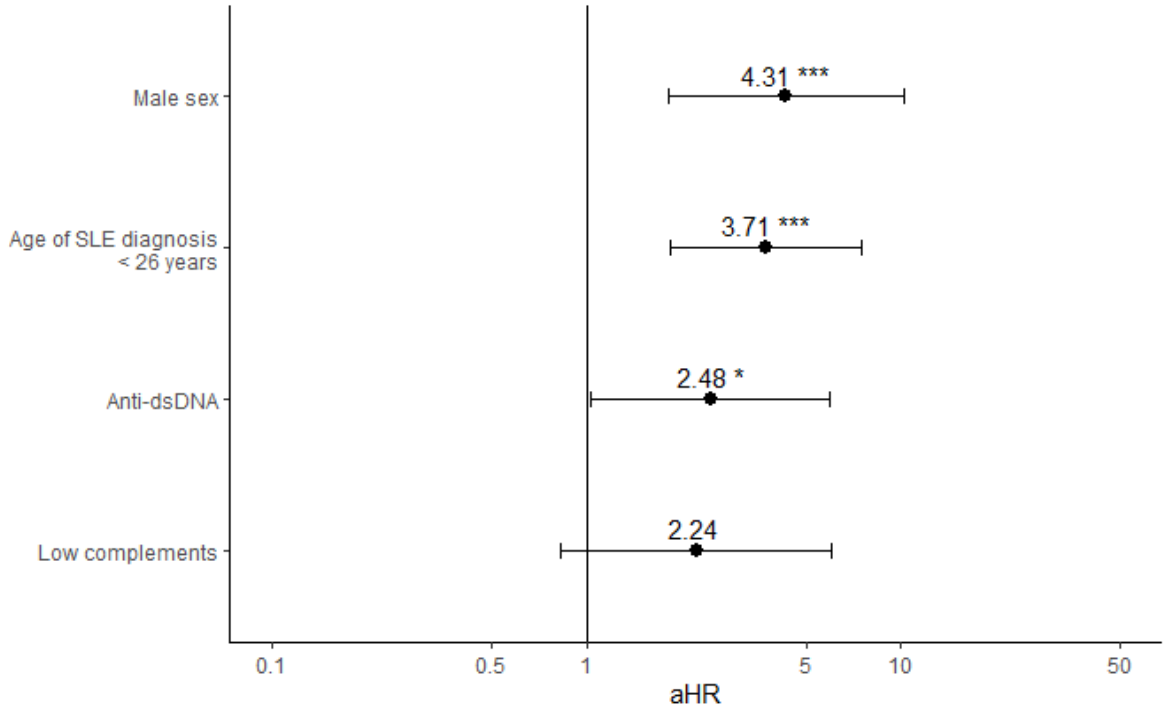

Figure 4.3.1: Factors at SLE diagnosis associated with subsequent kidney involvement. Multivariable Cox regression analysis for the identification of factors present at SLE diagnosis independently associated with subsequent kidney involvement: forest plot illustrating the adjusted hazard ratios and their corresponding 95% confidence intervals for each covariate; *: p-value < 0.05; ***: p-value < 0.001.

Characteristic	HR[1]	95% CI[1]	p-value
Sex			
Female	—	—	
Male	4.31	1.82, 10.2	**<0.001**
Age of SLE Dx < 26 yrs			
No	—	—	
Yes	3.71	1.84, 7.48	**<0.001**
Anti-dsDNA			
No	—	—	
Yes	2.48	1.03, 5.97	**0.043**
Low C3 and/or C4			
No	—	—	
Yes	2.24	0.83, 6.05	0.11

[1] HR = Hazard Ratio, CI = Confidence Interval

Table 4.3.2. *Cox PH multivariate regression with age as categorical for the outcome of LN.*

4.3.3. Interpretation of the results – across groups comparisons

In order to assess the combined impact of identified variables, we contrasted the aHR with the reference category, in the presence of an increasing number of predictive factors at baseline. The different linear combinations are presented in **Table 4.3.3.**

Notably, the collective aHR for all factors was almost 90-fold (88.77), when compared to a serologically inactive, older, female patient, thereby highlighting the crucial role of each independent factor on eventual kidney involvement, also illustrated in the 3-D bar plot in **Figure 4.3.2**.

Low_complements	Anti-dsDNA antibody	Age of SLE diagnosis	Sex	LN Hazard Ratio	95% CI	
No	No	>26	Female	1	1	1
Yes	No	>26	Female	2.24	0.829	6.05
No	Yes	>26	Female	2.48	1.029	5.96
Yes	Yes	>26	Female	5.54	1.811	16.98
No	No	<26	Female	3.71	1.843	7.48
Yes	No	<26	Female	8.31	2.555	27.01
No	Yes	<26	Female	9.19	3.321	25.45
Yes	Yes	<26	Female	20.58	6.226	68.02
No	No	>26	Male	4.31	1.817	10.24
Yes	No	>26	Male	9.66	2.694	34.61
No	Yes	>26	Male	10.69	3.104	36.78
Yes	Yes	>26	Male	23.92	6.021	95
No	No	<26	Male	16.01	4.562	56.19
Yes	No	<26	Male	35.84	7.684	167.15
No	Yes	<26	Male	39.66	9.211	170.75
Yes	Yes	<26	Male	88.77	18.745	420.41

Table 4.3.3. *Linear combinations of baseline variables and the corresponding LN HR, after multivariate Cox regression.*

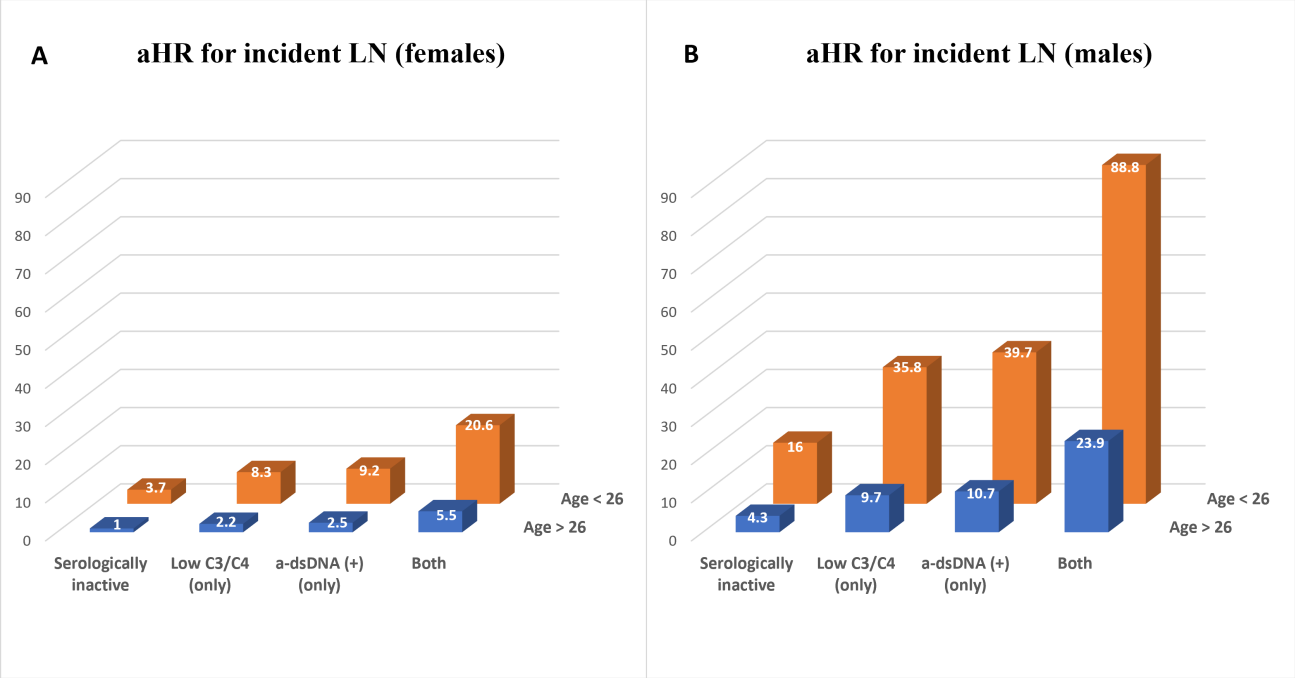

Figure 4.3.2: Combination of predictive factors progressively multiplies the risk of subsequent kidney involvement. Cox regression analysis estimates of the adjusted HR (aHR) for different combinations of serologic activity and age at SLE diagnosis for female (A) and male (B) patients, compared to a serologically inactive, female patient, with age at SLE diagnosis > 26 years, as reference.

4.4. Independent validation – Crete Lupus Registry

The findings of our primary analysis were externally evaluated using data from an independent cohort of 506 lupus patients from the Crete Lupus Registry. In this validation dataset, 46 patients developed kidney involvement, of whom 28 had nephritis at the time of SLE diagnosis.

4.4.1. Kaplan-Meier curves

KM curves across groups of interest for the original and validation cohorts were closely aligned, indicating that the overall LN incidence patterns were similar in the two populations (**Figure 4.4.1**). Age <26 years, male sex, anti-dsDNA and low complements were associated with significantly higher-incidence curves and retained a consistently higher LN risk for the duration of the study, similarly to the original cohort. Again, combined serologic activity at baseline

conferred the highest LN risk over time (**Figure 4.4.2**), but no significant interaction between them was detected.

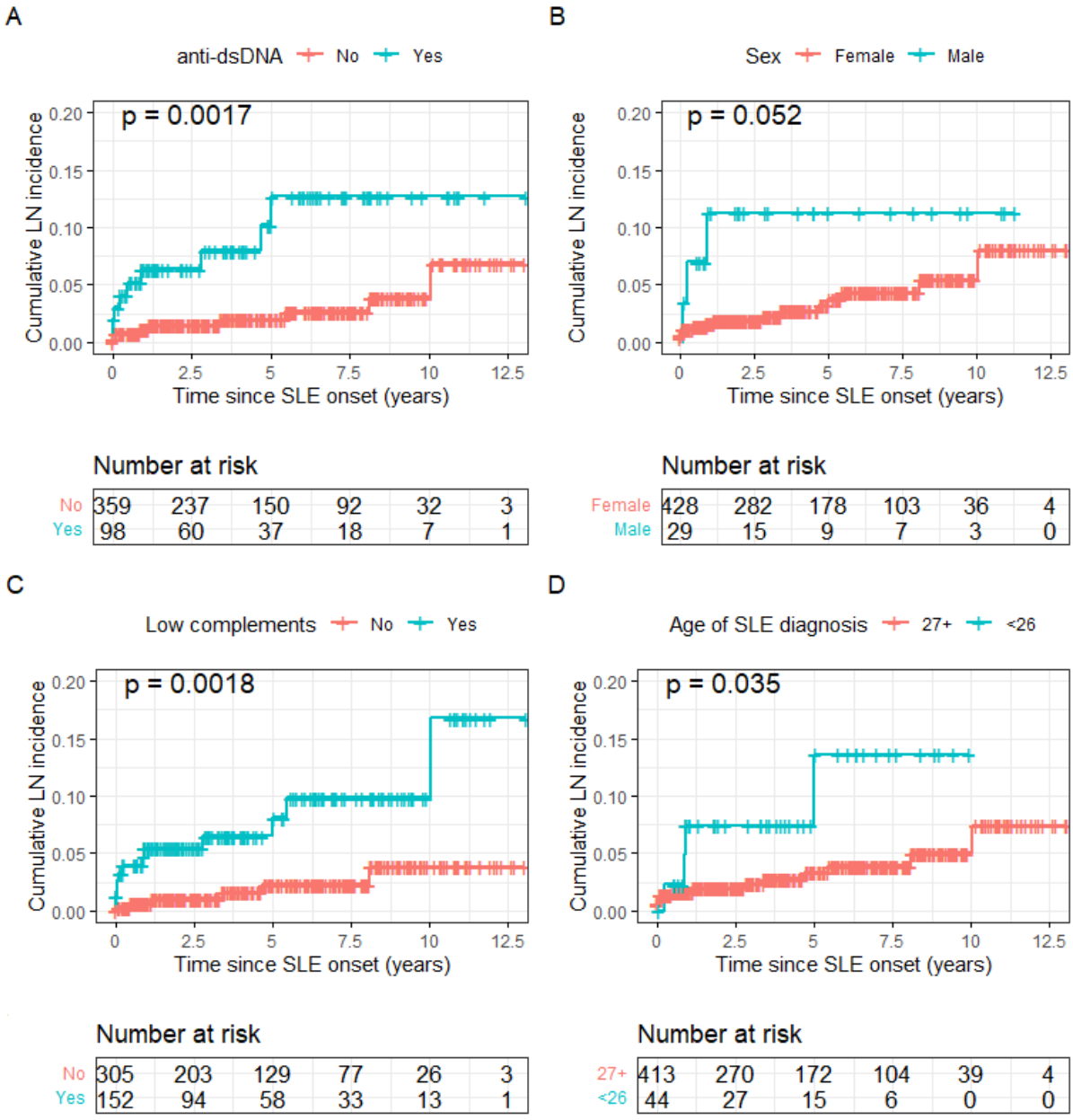

Figure 4.4.1. Composite cumulative incidence curves for incident kidney involvement across groups of interest for the validation cohort (Crete Lupus registry). Cumulative incidence curves comparing distinct groups within the validation cohort, based on (A) anti-dsDNA status, (B) sex, (C) complements status and (D) age category. P-values refer to the corresponding log-rank test.

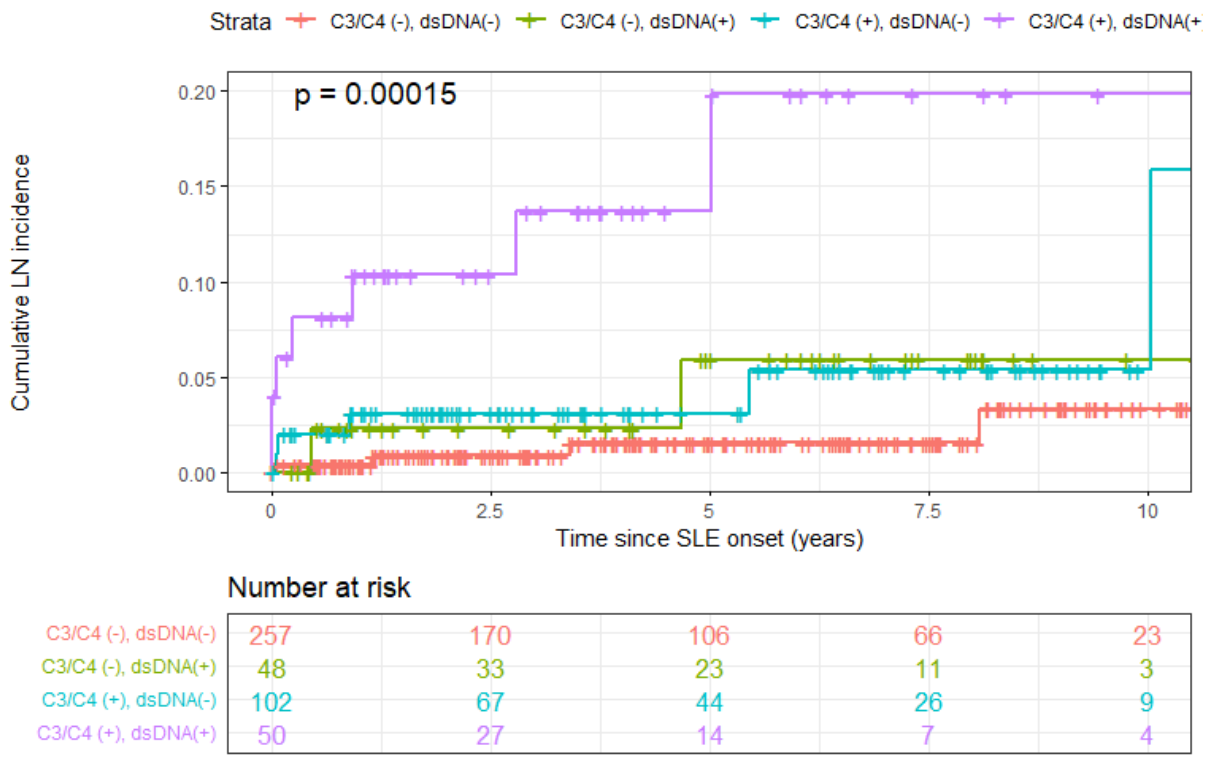

Figure 4.4.2. Risk stratification by baseline serologic activity profile in the validation cohort (Crete Lupus registry). Distinct cumulative incidence curves, representing various combinations of baseline anti-dsDNA antibody and complements status, estimated by the Kaplan-Meier method, on the validation cohort. The associated log-rank test revealed a highly significant difference in LN probability among the different groups (p-value < 0.001).

4.4.2. Cox regression

Cox PH regression revealed overlapping hazard ratios between the two centres, suggesting that the significant factors identified in the primary cohort remain consistent (**Table 4.4.1**). However, the multivariate model on the validation set exhibited reduced statistical significance and smaller magnitude of the associated effects, likely attributable to the smaller number of LN events,. More specifically, baseline anti-dsDNA antibody (HR 2.81, 95% CI: 1.06-7.43) and low complements (HR 3.20, 95% CI: 1.15-9.92) were significantly associated with an increased risk for LN, whereas male sex (HR 2.11, 95% CI: 0.58-7.66) and age <26 years (HR 1.42, 95% CI: 0.41-4.86) did not reach statistical significance in the validation cohort. In conclusion, findings from the Cox regression analysis were generally in line with the original cohort, supporting the generalizability of the identified LN risk factors.

Table 4.4.1. Multivariate Cox proportional hazards model results in the validation cohort (Crete Lupus registry)

Characteristic	**HR**[1]	**95% CI**[1]	**p-value**
Sex			
Female	—	—	
Male	2.11	0.58, 7.66	0.3
Age of SLE Dx < 26 years			
No	—	—	
Yes	1.42	0.41, 4.86	0.6
Anti-dsDNA			
No	—	—	
Yes	2.81	1.06, 7.43	**0.038**
Low C3 and/or C4			
No	—	—	
Yes	3.20	1.15, 8.92	**0.027**
[1] HR = Hazard Ratio, CI = Confidence Interval			

Footnote: Estimated hazard ratios with the associated 95% confidence intervals and p-values, after multivariate analysis on the validation cohort. The results were observed to overlap and align in the same direction with the original cohort data.

4.4.3. Model Performance Assessment on independent data

We further assessed the performance of our model by calculating Harrell's C-index in both cohorts. In the original cohort, the C-index of 0.768 indicated high discrimination ability. This finding suggests that our model could effectively rank patients according to their predicted risk. When applied to the validation cohort, the C-index remained remarkably stable (0.724), signifying that the model's ability to identify high-risk patients generalized well on independent data.

Furthermore, time-dependent AUC values obtained through cross-validation ranged between 0.78 and 0.88 (**Figure 4.4.3**). In essence, the validation findings suggest that the model developed on the 'Attikon' cohort maintained robust accuracy across many time points, when applied to external cohort.

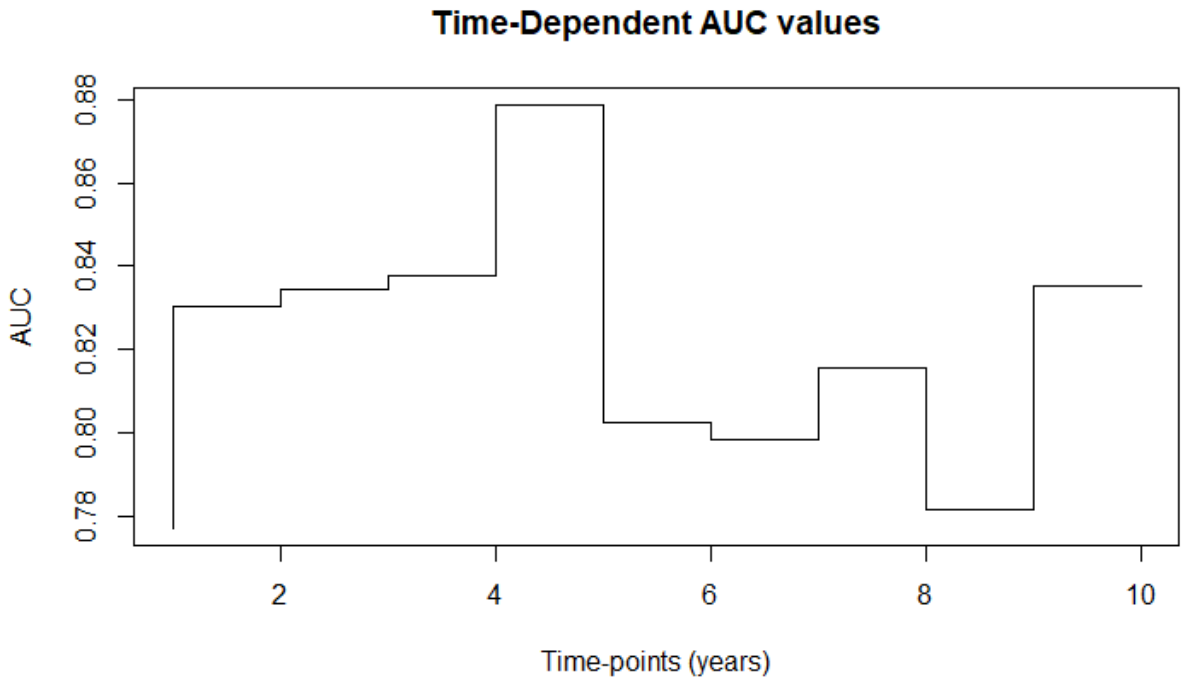

Figure 4.4.3. Cross-validation AUC values for predictive accuracy of the original model, applied to independent cohort data. Extension of AUC that accounts for time-to-event data, where sensitivity and specificity are based on the survival time under investigation. The observed AUC values (ranging from 0.78 to 0.88) at different time-points pertain to the application of the original model on the independent cohort data, indicating a high level of predictive accuracy during external validation.

4.5. Competing risks analysis: proliferative versus non-proliferative LN

To explore whether baseline factors identified in the previous analyses were associated with specific histologic class, a competing risks analysis was conducted in patients from the 'Attikon' cohort who underwent kidney biopsy and had proliferative (n = 35) versus non-proliferative LN (n = 24). Non-parametric cumulative incidence curves, estimated by the Aalen-Johansen estimator, demonstrated similar temporal patterns for the two histologic groups, when stratified based on genderm anti-dsDNA and low complements status (**Figure 4.5.1**). However, the cumulative incidence curves according to age group did not seem to significantly differ when examining non-proliferative LN cases, but only for proliferative LN. It should be noted that due to the method used, these curves do not represent a 'proper' LN risk distribution, but a subdistribution function, which cannot be properly interpreted as a realizable quantity. They do, however, allow us to make assess the associations with previously identified groups.

After employing *cause-specific Cox PH models*, predictive baseline factors were generally consistent, with *overlapping confidence intervals* for the hazard ratios but of smaller magnitude compared to the original cohort data. Additionally, more robust predictor-outcome relationships were observed for proliferative compared to non-proliferative LN (**Tables 4.5.1** and **4.5.2**).

- For proliferative LN significant associations were found with age <26 years (HR 6.96, $p < 0.001$), anti-dsDNA (HR 6.59, $p < 0.001$) and low complements (HR 8.15, $p < 0.01$) on univariate analysis. On multivariate analysis male sex (aHR 3.96, $p < 0.05$) and age <26 years (aHR 5.93, $p < 0.001$) were significant risk factors.
- For non-proliferative LN the associations were less prominent, with only anti-dsDNA reaching a statistically significant HR of 3.77 ($p < 0.05$). Nevertheless, a trend for greater LN risk was found for all previously identified risk factors, in line with the primary findings.

It is important to acknowledge that these analyses were probably underpowered, as a consequence of dividing the outcome into two competing events. This limitation may have hampered the identification of significant associations, especially for non-proliferative LN.

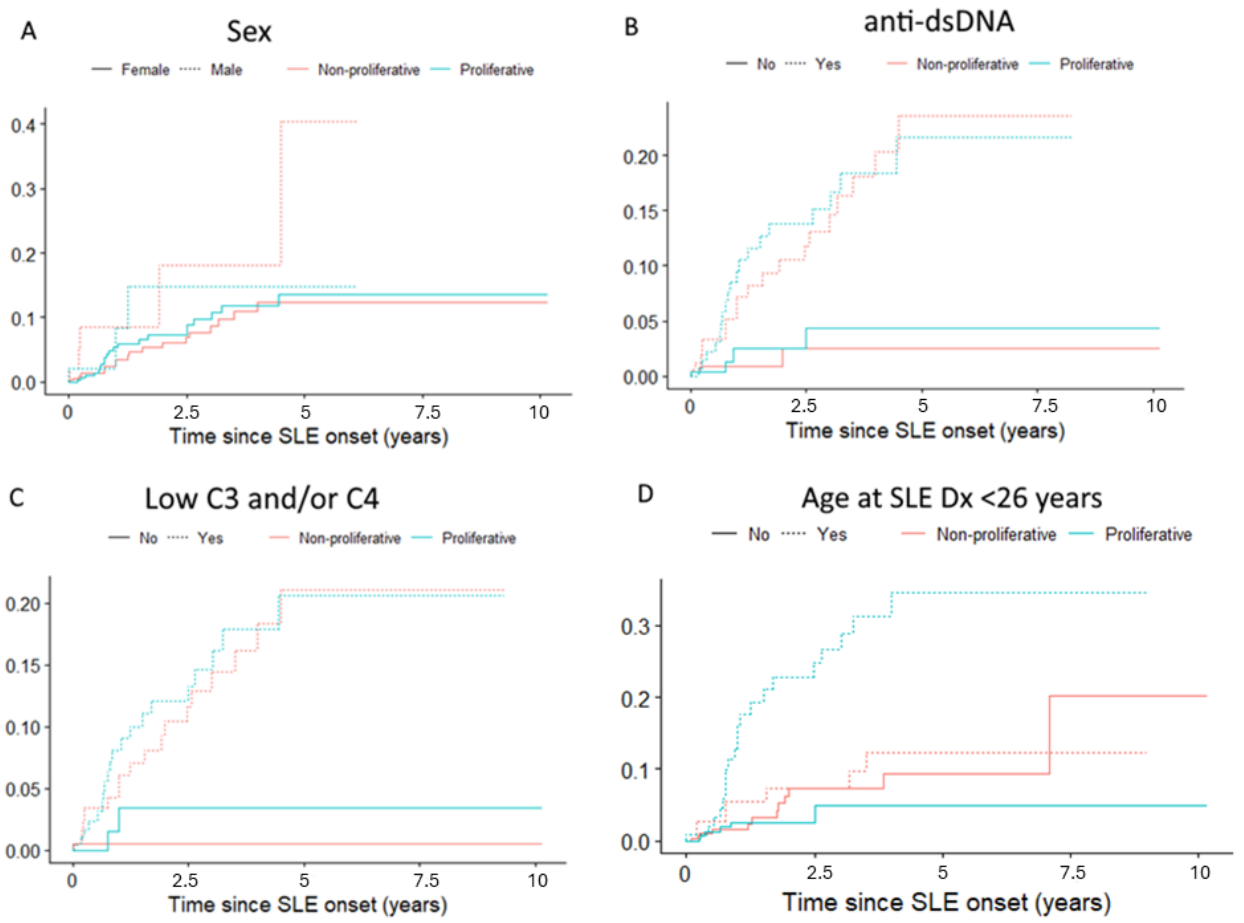

Figure 4.5.1: Cumulative incidence curves for proliferative and non-proliferative LN. Cumulative incidence curves for the two possible outcomes of proliferative and non-proliferative LN, estimated by the Aalen-Johansen method, based on sex (A), anti-dsDNA (B), complements status (C) and age group (D).

Table 4.5.1 Cause-specific Cox regression for *proliferative* LN

	Univariate			Multivariate		
Characteristic	**HR[1]**	**95% CI[1]**	**p-value**	**HR[1]**	**95% CI[1]**	**p-value**
Sex						
Female	—	—		—	—	
Male	1.93	0.67, 5.53	0.22	3.96	1.30, 12.1	**0.015**
Age of SLE Dx <26 years						
No	—	—		—	—	
Yes	6.96	2.96, 16.4	**<0.001**	5.93	2.26, 15.6	**<0.001**
Anti-dsDNA						
No	—	—		—	—	
Yes	6.59	2.30, 18.9	**<0.001**	3.02	0.84, 10.8	0.091
Low C3 and/or C4						
No	—	—		—	—	
Yes	8.15	1.93, 34.3	**0.004**	3.33	0.74, 14.9	0.12

[1] HR = Hazard Ratio, CI = Confidence Interval

Footnote: Proliferative-specific Cox PH regression, showing associations after of univariate (left) and multivariate (right) analysis, with accompanying 95% confidence intervals and p-values.

Table 4.5.2 Cause-specific Cox regression for *non-proliferative* LN

	Univariate			Multivariate		
Characteristic	**HR[1]**	**95% CI[1]**	**p-value**	**HR[1]**	**95% CI[1]**	**p-value**
Sex						
Female	—	—		—	—	
Male	2.25	0.66, 7.71	0.20	2.18	0.47, 10.2	0.3
Age of SLE Dx <26 years						
No	—	—		—	—	
Yes	1.68	0.68, 4.12	0.26	1.12	0.36, 3.51	0.8
Anti-dsDNA						
No	—	—		—	—	
Yes	3.77	1.24, 11.5	**0.020**	1.96	0.57, 6.75	0.3
Low C3 and/or C4						
No	—	—		—	—	
Yes	2.97	0.85, 10.4	0.089	1.70	0.43, 6.63	0.4

[1] HR = Hazard Ratio, CI = Confidence Interval

Footnote: Non-proliferative-specific Cox PH regression, showing associations after of univariate (left) and multivariate (right) analysis, with accompanying 95% confidence intervals and p-values.

5. Discussion

LN is a major milestone in the natural history of SLE. Together with major neuropsychiatric disease, it impacts on the prognosis of patients more than any other manifestation, given also its relatively increased frequency compared to other serious manifestations. Indeed, despite declining rates over recent decades, up to 20% of LN patients may progress to end-stage kidney disease within the first decade of the disease course, thus prompting for early identification with the first signs of kidney involvement (Tektonidou, Dasgupta and Ward, 2016). To this end, while increased awareness is needed for all patients with SLE, it is important to identify early those subsets of patients who are at particularly increased risk to develop LN. We used data from the 'Attikon" lupus cohort and found that, at disease onset, male patients, those younger in age, and those with serologic activity carry a higher risk to manifest evidence of kidney involvement at some point in the future. Importantly, a combination of these factors confers a multiplicative, significantly higher risk over individual factors alone. Finally, we were able to confirm our findings in a totally independent large patient cohort, further strengthening the validity of the results.

The cumulative incidence of LN in our cohort consisting exclusively of white patients was 22%, in line with existing literature that supports a lower incidence of kidney disease in the white race compared to African-Americans, Asians or Hispanics (rates ranging from 35 to well over 50%, depending on several cohort studies) (Seligman et al., 2002;Feldman et al., 2013; Hanly et al., 2015; Jakes et al., 2012). Importantly, we also confirmed that, although LN is frequently evident as a presenting manifestation in a substantial proportion of SLE patients, more than half of LN patients (53%) developed kidney involvement following SLE diagnosis. Median time of LN occurrence after SLE diagnosis was 4 years, while a considerable number of patients (n = 21) were diagnosed with LN more than 5 years from diagnosis, which is traditionally considered as a timepoint after which LN rarely develops. Thus, vigilance for emerging signs of kidney involvement should be lifelong in SLE, and late-onset LN (i.e., diagnosed more than 5 years following disease onset) has similar clinical characteristics and outcome with its early-onset counterpart (Ahn et al., 2020;Delfino, Dos Santos and Skare, 2020).

When LN is not part of the initial clinical presentation of SLE, identifying patient subgroups that are at particularly increased risk to subsequently develop kidney disease is of utmost importance. Of note, most studies that have examined risk factors for LN have not separated LN cases based

on timing of its appearance Our findings corroborate evidence from previous literature which support that patients with SLE of younger age and male sex are more likely to develop LN (Seligman et al., 2002; Hanly et al., 2015). Our analysis identified a cut-off for age at SLE diagnosis (26 years), below which the risk for future kidney involvement increases significantly. Moreover, acute changes in serologic activity (sharp rises of anti-ds DNA or acute drops in C3/C4 levels) have traditionally been linked to imminent flares of LN, however their status at baseline has been less studied as a risk factor for LN occurrence later in the course of the disease. Two recent studies in Asian populations found that hypocomplementemia and high anti-dsDNA titre at diagnosis of SLE were associated with a risk of future LN, although multivariate analysis found an independent association only for low C3 in one of the two studies (Kwon *et al.*, 2018). Our findings are in line with these observations and suggest that baseline serologic activity is a major determinant of future kidney disease, while the latter showed no association with any of the clinical manifestations during disease presentation. More importantly, our study further suggests that the type of serologic activity matters, because patients with combined anti-ds DNA positivity and low C3 and/or C4 were at significantly higher risk to exhibit kidney involvement over their disease course, compared to either positivity alone.

Lupus patients who present with 'high-risk' features to develop severe disease represent a challenge for treating physicians. Patients with persistent serologic activity are at increased risk for a subsequent disease flare and warrant close monitoring (Huang et al., 2021; Ng et al., 2006), yet there is wide consensus that sole serologic activity is not an indication for treatment intensification in patients with lupus(Steiman *et al.*, 2012), and the most recent widely accepted definition of remission from the Definition Of Remission In SLE (DORIS) international project does not require serologic inactivity for a patient to be labelled as being in remission (van Vollenhoven *et al.*, 2021). Our study is focused solely on baseline characteristics (when serologic is typically also accompanied by clinical activity, hence therapy is needed) and does not relate to later stages of the disease when hypocomplementemia and/or anti-ds DNA positivity may be the only finding. Nevertheless, the issue of treatment of patients at high risk to develop later serious manifestations is important. The recent EULAR recommendations advocate for an early use of disease-modifying drugs, including biologics, to better control the disease (Fanouriakis *et al.*, 2023). A recent post-hoc analysis of the BLISS trials reported that belimumab may be able to reduce the incidence of de novo renal flares (Parodis *et al.*, 2023), a formal pragmatic randomised

clinical trial would be needed to address this question. Based on our study findings, patients at the highest risk, as suggested by our findings, would be particularly suitable candidates for such a trial. Our study has several limitations that need to be acknowledged. First, both the 'Attikon' and the validation cohort consist almost exclusively of Whites, thus data cannot necessarily be extrapolated to populations with different racial characteristics. Confirmation of our findings in high-risk groups, such as African-American or Asian patients, would be desirable. Also, we have not included in our analysis the therapies that patients received after SLE diagnosis but prior to the development of LN, because specific time periods of individual immunosuppressive treatments are not captured in the dataset of the 'Attikon'cohort. Thus, potential differences in immunosuppressive treatment and/or use of hydroxychloroquine which may have influenced the development of LN could not be taken into account. Moreover, complement and anti-ds DNA antibody levels tend to fluctuate over time and be affected by administered therapies. Based on data availability, we focused only on baseline values of these tests at the time of SLE diagnosis and were not able to examine possible longitudinal changes and their potential to alter their association with LN occurrence. Finally, in our dataset, both C3 and C4 are documented as a single binary variable in our database (low C3 and/or C4 vs both being normal), thus we could not decipher whether either of, only one of, or both low C3 and C4 at SLE diagnosis are independently associated with risk of incident LN.

In conclusion, our retrospective cohort study in an exclusively white race SLE cohort with external validation showed that patients who are diagnosed at a young age and have evidence of combined serologic activity -especially combined hypocomplementemia and high anti-ds DNA- are at substantially increased risk to develop kidney involvement over the following years. These patients clearly represent a special high-risk population who should be put under vigilant monitoring for the earliest detection of a disease manifestation with profound prognostic repercussions.

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Abstract

Objectives: To discern predictive factors for incident kidney involvement in patients with systemic lupus erythematosus (SLE).

Methods: Patients with SLE from the 'Attikon' Lupus cohort were monitored for lupus nephritis (LN), defined by kidney histology and/or classification criteria. Demographic and clinical characteristics at baseline were compared against patients who did not develop LN. LN-free survival curves were generated by Kaplan-Meier. A multivariate Cox proportional hazards model was used to identify independent predictors of LN. Independent validation was performed in the University of Crete Lupus registry.

Results: Among the 570 patients in the derivation cohort, 59 exhibited LN as their initial presentation, while an additional 66 developed LN during the follow-up period (collectively, 21.9% incidence). In the latter group, baseline factors predictive of subsequent kidney involvement were male sex (multivariable-adjusted [a]HR 4.31, 95% CI: 1.82-10.2), age of SLE diagnosis below 26 years (aHR 3.71, 95% CI: 1.84-7.48), high anti-dsDNA titre (aHR 2.48, 95% CI: 1.03-5.97) and low C3 and/or C4 (although not statistically significant, aHR 2.24, 95% CI: 0.83-6.05, $p = 0.11$). A combination of these factors at time of diagnosis conferred an almost 90-fold risk compared to serologically inactive, older, female patients (aHR 88.77, 95% CI:18.75-420.41), signifying a very high-risk group. Independent validation in the Crete Lupus registry showed concordant results with the original cohort.

Conclusion: Male sex, younger age and serologic activity at SLE diagnosis are strongly associated with subsequent kidney involvement. Vigilant surveillance and consideration of early use of disease-modifying drugs is warranted in these subsets of patients.

Περίληψη

Σκοπός: Η ανάδειξη παραγόντων κινδύνου για μελλοντική εμφάνιση νεφρικής προσβολής σε ασθενείς με Συστηματικό Ερυθηματώδη Λύκο (ΣΕΛ).

Μέθοδοι: Ασθενείς από την κοόρτη ασθενών με ΣΕΛ του Π.Γ.Ν. 'Αττικόν' παρακολουθήθηκαν αναδρομικά μέχρι την εμφάνιση νεφρίτιδας, διαγνωσμένη βάση νεφρικής βιοψίας ή/και κριτηρίων ταξινόμησης. Συλλέχθηκαν δημογραφικά και κλινικο-εργαστηριακά αρχικά χαρακτηριστικά και έγινε σύγκριση μεταξύ ασθενών που εμφάνισαν ή όχι νεφρίτιδα. Σχεδιάστηκαν καμπύλες επιβίωσης και αθροιστικής επίπτωσης νεφρίτιδας με την Kaplan-Meier μέθοδο. Μονοπαραγοντικά και πολυπαραγοντικά μοντέλα Cox χρησιμοποιήθηκαν για την εντόπιση ανεξάρτητων παραγόντων κινδύνου για νεφρίτιδα. Πραγματοποιήθηκε ανεξάρτητη αξιολόγηση των ευρημάτων στην κοόρτη ασθενών με ΣΕΛ του Π.Γ.Ν. Ηρακλείου.

Αποτελέσματα: Από τους 570 ασθενείς της βασικής κοόρτης, οι 59 παρουσίαζαν νεφρίτιδα ως αρχική εκδήλωση της νόσου και 66 ανέπτυξαν νεφρίτιδα κατά τη διάρκεια της μελέτης (αθροιστική συχνότητα 21.9%). Οι παράγοντες κινδύνου που συσχετίσθηκαν με ακόλουθη εμφάνιση νεφρικής προσβολής ήταν το ανδρικό φύλο (multivariable-adjusted [a]HR 4.31, 95% CI: 1.82-10.2), η ηλικία διάγνωσης ΣΕΛ < 26 ετών(aHR 3.71, 95% CI: 1.84-7.48), υψηλά επίπεδα anti-dsDNA αντισωμάτων (aHR 2.48, 95% CI: 1.03-5.97) και τα χαμηλά συμπληρώματα C3 ή και C4 (παρόλο που δεν έφτασαν στατιστική σημαντικότητα, aHR 2.24, 95% CI: 0.83-6.05, p = 0.11). Ο συνδυασμός των παραπάνω παραγόντων κατά τη διάγνωση του ΣΕΛ σχετίσθηκε με σχεδόν 90-πλάσιο κίνδυνο για ανάπτυξη νεφρίτιδας, συγκριτικά με ορολογικά ανενεργούς, >26 ετών ασθενείς γυναικείου φύλου (aHR 88.77, 95% CI:18.75-420.41), αναδεικνύοντας ένα γκρουπ ασθενών ιδιαίτερα αυξημένου κινδύνου. Η εξωτερική αξιολόγηση των ευρημάτων στην κοόρτη ασθενών με λύκο του ΠΑΓΝΗ ανέδειξε σύμφωνα αποτελέσματα με την αρχική κοόρτη.

Συμπεράσματα: Το ανδρικό φύλο, η ηλικία <26 ετών με ορολογική ενεργότητα κατά τη διάγνωση του ΣΕΛ συσχετίσθηκαν σε στατιστικά σημαντικό βαθμό με ακόλουθη εμφάνιση νεφρικής προσβολής. Η στενή επιτήρηση και ενδεχόμενη πρώιμη έναρξη ανοσοτροποποιητικής αγωγής ενδείκνυται ιδιαίτερα σε αυτές τις κατηγορίες ασθενών.

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Supplement

Supplemental Table S1. *The new EULAR/ACR 2019 classification criteria for SLE.*

Entry criterion **Antinuclear antibodies (ANA) at a titer of ≥1:80 on HEp-2 cells or an equivalent positive test (ever)**			
↓			
If absent, do not classify as SLE If present, apply additive criteria			
↓			
Additive criteria **Do not count a criterion if there is a more likely explanation than SLE.** **Occurrence of a criterion on at least one occasion is sufficient.** **Criteria need not occur simultaneously.** **Within each domain, only the highest weighted criterion is counted toward the total score.**			
Clinical domains and criteria	**Weight**	**Immunology domains and criteria**	**Weight**
Constitutional	2	***Antiphospholipid antibodies***	
Fever		Anti-cardiolipin antibodies OR	
Hematologic	3	anti-β2GP1 antibodies OR	2
Leukopenia		lupus anticoagulant	
Thrombocytopenia	4	***Complement proteins***	
Autoimmune hemolysis	4	Low C3 OR low C4	3
		Low C3 AND low C4	4
Neuropsychiatric	2		
Delirium		***SLE-specific antibodies***	
Psychosis	3	Anti-dsDNA antibody* OR	
Seizure	5	Anti-Smith antibody	6
Mucocutaneous			
Non-scarring alopecia	2		
Oral ulcers	2		
Subacute cutaneous OR discoid lupus	4		
Acute cutaneous lupus	6		
Serosal			
Pleural or pericardial effusion Acute pericariditis	6		
Musculoskeletal			
Joint involvement	6		
Renal			
Proteinuria >0.5g/24h	4		
Renal biopsy Class II or V lupus nephritis	8		
Renal biopsy Class III or IV lupus nephritis	10		
	Total score:		
SLE classification requires: **(a) fulfilled entry criterion, (b) at least one clinical criterion and (c) total score ≥ 10 points.**			