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# Are lupus patients characterized by a phenomenon of accelerated aging? Analysis of literature and pilot study on epigenetic biomarkers of age.

Noémie Gensous

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**Are lupus patients characterized by a phenomenon of  
accelerated aging?**

**Analysis of literature and pilot study on epigenetic biomarkers of age.**

Sous la direction de Monsieur le Pr. Pierre DUFFAU

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Monsieur le Pr. Patrick BLANCO  
Monsieur le Pr. Pierre DUFFAU  
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## ***1. Introduction***

Systemic lupus erythematosus (SLE) is a chronic systemic autoimmune disease characterized by a breakdown of tolerance against nuclear antigens, leading to autoantibody production, immune complex formation and organ damage (Tsokos 2011). Thanks to improvements during the last decades in diagnosis, therapeutics (immunosuppressive agents, biotherapies) and medical care (improved management of end stage renal and cardiovascular diseases especially), the lifespan of SLE patients has remarkably increased. While the five-year survival rates were about only 50% in the 1950's, they nowadays exceed 90% in SLE patients (95% from 2008 to 2016 in adults from high-income countries) (Tektonidou et al. 2017). However, despite the important increase in lifespan, standardized mortality ratio (SMR) (ratio of the number of deaths observed in a given cohort divided by the deaths reported in the general population for the same time period) are still high (Tselios et al. 2019; Lee et al. 2016; Yurkovich et al. 2014; Bernatsky et al. 2006; Scherlinger et al. 2020; Barber et al. 2021). Some clinical manifestations more commonly found during old age, such as cardiovascular (CV) events, renal disease, infections or cancers, appear frequently at a younger age in SLE patients, considered either as sequelae of the disease course or consequences of medications, and are responsible for increased mortality and morbidity in this population (Nossent et al. 2007; Thomas et al. 2014).

Thus, it is well established that CV events occur more frequently in patients with chronic inflammatory conditions, including SLE, as compared to the general population (Skaggs et al. 2012). The increased risk of CV diseases (CVD) in SLE patients is known since the 1970's, particularly with the initial publication of Urowitz *et al.* which described a bimodal mortality pattern in SLE, with important incidence of deaths associated with myocardial infarction in patients who die late in the course of their disease (Urowitz et al. 1976). Since this date, many studies have evaluated the incidence and epidemiology of CVD in patients with SLE, and have

confirmed that subclinical atherosclerosis and CVD occur more frequently and with an earlier onset in this population as compared to healthy individuals (Reviewed in (Skaggs et al. 2012; Schoenfeld et al. 2013; Liu & Kaplan 2018; Giannelou & Mavragani 2017; Lu et al. 2021)). The observed increased risk of CVD in SLE is the result of the combination of traditional CV risk factors and SLE-specific ones. Indeed, traditional CV risk factors (such as hypertension, smoking, diabetes or hypercholesterolemia) do not fully explain the high rates of CVD reported so far in SLE (Roman et al. 2003; Esdaile et al. 2001). Other several factors that are inherent to SLE itself have been described as contributors to the pathogenesis of premature and accelerated atherosclerosis in this disease. They include, amongst others, older age at diagnosis, longer disease duration of SLE, activity and chronicity, higher levels of SLE-associated damage, high cumulative glucocorticoid dosage, increased thrombosis in the setting of antiphospholipid antibodies. From a cellular and molecular point of view, they rely on specific inflammatory mediators and immune dysregulation, leading to chronic inflammation (Skaggs et al. 2012; Liu & Kaplan 2018; Giannelou & Mavragani 2017; Manzi et al. 1997; Roman et al. 2007; Roman et al. 2003; Doria et al. 2003).

Infections are also a leading cause of morbidity and mortality in the SLE population (Bernatsky et al. 2006; Yurkovich et al. 2014; Lee et al. 2016). Infections rates (especially regarding infections of the respiratory tract) are found at increased frequencies and with higher severity in the population of SLE patients (Danza & Ruiz-Irastorza 2013; Caza et al. 2014). Multiple factors contribute to the vulnerability to infections of this population, both related to the autoimmune disease itself and its imbalanced immune responses, but also regarding the use of certain treatments (especially high doses of glucocorticoids and immunosuppressive drugs). In addition to the increased susceptibility to infectious diseases, SLE population is also characterized by poorer vaccinations responses. Thus, according to recent systematic literature reviews and meta-analyses, while the safety profile of vaccines appeared to be good in SLE

patients, immunogenicity of some vaccines (against particular viral influenza strains for example) seems to be reduced in SLE patients as compared to the general population (Pugès et al. 2016; Rondaan et al. 2019; Huang et al. 2016; Izmirly et al. 2021).

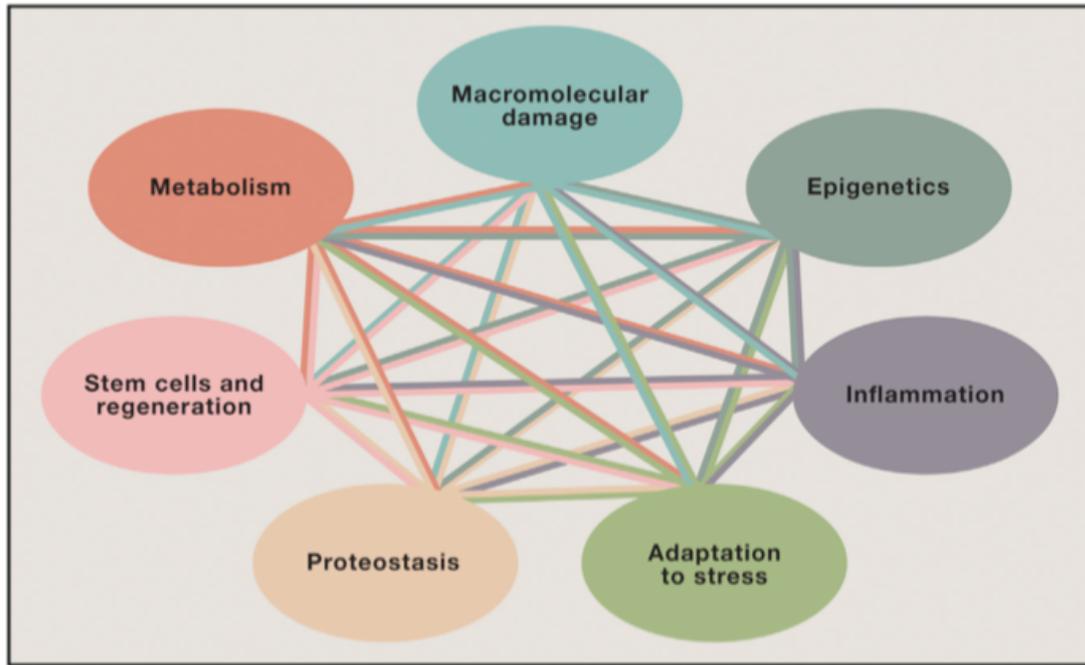
Finally, different studies have indicated an increased susceptibility to certain types of cancer in individuals with SLE, especially for hematologic malignancies (predominantly non-Hodgkin's lymphoma), lung or cervical cancers (Reviewed in (Gayed et al. 2009; Goobie et al. 2015)). Several hypotheses have been suggested to explain the mechanisms responsible for the association between cancer and SLE, including genetic predispositions common to autoimmunity and hematologic malignancies, defects in the humoral and cellular immune systems responsible for tumor immunosurveillance, and increased influence of conventional cancer risk factors such as smoking. Except for cervical cancers, the impact of immunosuppressive therapies on the occurrence of malignancies appears controversial (Gayed et al. 2009; Bernatsky et al. 2005). Despite the increased incidence of cancer in the SLE population, it is to be noted that regarding cancer-related mortality, two recent meta-analyses reported that the risk of death related to malignancies was not significantly increased in SLE patients (Lee et al. 2016; Yurkovich et al. 2014).

As a consequence of the increased lifespan in SLE, more and more lupus patients are reaching an advanced age with longer disease duration. A large proportion of this population is getting to face early in life important co-morbidities, which are, for certain of them, more frequently found in older adults (*i.e.* age-related diseases), raising the question of the presence of an acceleration of the aging process in SLE patients.

## ***2. SLE and the pillars of aging: analysis of literature***

### ***2.1 Aging and its pillars***

Aging can be defined as the normal, time-dependent and progressive decline of function, occurring at the cellular, tissue and organismal levels over the lifespan, ultimately resulting in death. Aging is a complex and multifaceted phenomenon, resulting of a complex interplay between genetic, environmental, and stochastic variables. It is considered as the result of the progressive accumulation of damage coupled with a decline in maintenance (Goldsmith 2012; Blagosklonny 2013; Kowald & Kirkwood 2016). Aging is recognized as a major risk factor for a wide range of non-communicable and chronic diseases, including cancers, cardiovascular and neurodegenerative diseases or metabolic disorders. Scientific community agrees that aging is characterized by specific hallmarks, which rely on well-defined biological pathways. According to recent conceptualizations, an attempt has been made to clearly define a small set of these basic molecular and cellular mechanisms, highly interconnected, on which aging relies on (Kennedy et al. 2014; López-Otín et al. 2013) (**Figure 1**). It is considered that age-related diseases rely on the same mechanisms and are deviations from the normal trajectory of aging (Kennedy et al. 2014; Franceschi et al. 2018).



**Figure 1:** The seven pillars of aging, according to Kennedy *et al.* (2014).

## ***2.2 SLE and the pillars of aging***

Given the increased prevalence of some age-related diseases observed in SLE patients, we wanted to evaluate if lupus was associated with features of acceleration of the mechanisms which underly aging. To this aim, in this section, we reviewed and compared literature evidence on the pillars of aging evaluated in SLE patients and explored possible interconnections between these two frameworks.

### **2.2.1 Macromolecular damage**

Accumulation of macromolecular damage throughout life, and especially DNA damage, is considered as a major contributor to aging (Moskalev *et al.* 2013; Soares *et al.* 2014). DNA is constantly exposed to endogenous and exogenous (physical, chemical and biological) agents that can stimulate a large variety of DNA lesions (point mutations, translocations, gene

disruption, double-stranded breaks, ...), both in nuclear and mitochondrial DNA. Different mechanisms are involved in the repair of these lesions (homologous recombination, nonhomologous end-joining, mismatch repairs, ...) and are collectively responsible for ensuring the integrity of DNA. However, while we age, both excessive macromolecular damage and declining efficiency of DNA repair systems favor the aging process. Notably, DNA damage accumulation can result in genomic instability and can promote malignant transformation, cellular senescence or cell death by apoptosis.

In SLE, multiple studies have demonstrated the presence of increased genomic DNA damage in patients as compared to healthy controls. Increased levels of damaged DNA have been observed in neutrophils (McConnell et al. 2002), low density granulocytes (Singh et al. 2014), cell samples from buccal mucosa (Al-Rawi et al. 2014) or circulating mononuclear cells (Souliotis & Sfikakis 2015) of SLE patients. Likewise, damage to mitochondrial DNA is more common in peripheral blood mononuclear cells (PBMC) of SLE patients as compared to healthy individuals, with a positive correlation between mitochondrial DNA lesions and disease duration (López-López et al. 2014). Repair mechanisms of DNA damage appear to be also impaired and less efficient with delayed kinetics in SLE patients, both in pediatric and adult subpopulations (Davies et al. 2012; McCurdy et al. 1997; Souliotis et al. 2016). In PBMC from adults SLE patients, DNA repair deficiencies were observed regardless of disease activity level and were found correlated with apoptosis rates (Souliotis et al. 2016).

Several polymorphisms in genes coding for mediators of crucial pathways for DNA repair have been associated with SLE (Lin et al. 2009; Jahantigh et al. 2015; Lin et al. 2010; Bassi et al. 2008; De Azevêdo Silva et al. 2014). Moreover, it has been demonstrated that accumulation of damaged DNA is also related to impaired function of some critical enzymes involved in DNA degradation, such as three prime repair exonuclease 1 (TREX1) (Yan 2017), RNaseH2 (Günther et al. 2015) or Dnase1 (Napirei et al. 2000).

Thereby, there is extensive evidence that increased DNA damage formation, defective repair and defective DNA degradation are important factors implicated in the pathogenesis of SLE. They play a crucial role in the early initiation and the progression of the disease, by the induction of increased rates of cell death, accumulation of cytosolic DNA and generation of neoantigens, that could trigger innate immune system activation and auto-antibodies production (Souliotis et al. 2019). Also, as it has been observed, for example, that damage of mitochondrial DNA correlate with disease duration (López-López et al. 2014), indicating that damage accumulate over the years, it could be hypothesized that these phenomena also participate to an acceleration of the aging process in SLE patients.

Some critical chromosomal regions are particularly susceptible to age-related accumulation of DNA damage: telomeres. Telomeres are repetitive DNA sequences, consisting of multiple TTAGGG repeats, located at the ends of the chromosomes, responsible for their stability and protection (Sfeir 2012). At each cell division, a certain number of nucleotides at these telomeric ends is lost, and telomers become slightly shorter at every DNA replication, ultimately leading to cell senescence. To compensate telomeric loss, some cells express the enzyme telomerase which is able to elongate telomeres by adding telomere repeats. However, telomerase activity declines with age, and telomere shortening still occurs progressively. Telomeric length is widely recognized as a powerful index of cellular aging and is probably one of the most studied biomarker of age (Jylhävä et al. 2017). Correlations between short telomeres and mortality risk, cancer, CVD risk or cognitive status, particularly at younger ages, have been well demonstrated (Jylhävä et al. 2017; Rode et al. 2015).

Several researchers have investigated telomere shortening in SLE. Collectively, these studies show that granulocytes, mononuclear leucocytes or lymphocytes of SLE patients have shorter telomeres when compared to age-matched healthy subjects, indicating a clear pattern of

accelerated senescence (Honda et al. 2001; Kurosaka et al. 2003; Kurosaka et al. 2006; Fasching et al. 2017; Haque et al. 2013; Hoffecker et al. 2013; Wu et al. 2007; Skamra et al. 2013). Interestingly, this difference is even more pronounced in younger age groups (Honda et al. 2001; Kurosaka et al. 2003; Fasching et al. 2017; Wu et al. 2007). No association between telomere length and disease activity or disease duration was identified (Haque et al. 2013). Wu *et al.* quantified the telomere shortening observed in SLE patients and tried to convert it in terms of chronological time. According to their study, the average reduction in telomere length in SLE patients was equivalent to a premature senescence of 16.5 years in mononuclear cells and of 13.4 years in polymorphonuclear neutrophils (Wu et al. 2007). Different attempts to use telomere length as a marker of CV risk in SLE patients were performed. Shorter telomere lengths were observed in young SLE women with carotid plaque (Skamra et al. 2013). On the contrary and counterintuitively, Haque *et al.* observed that carotid plaque was particularly prevalent in patients with the higher tertile of telomere length (Haque et al. 2013). The explanation for this observation is unclear. The cross-sectional design of the study could have led to a censorship of data, as the authors discuss in the publication. Patients with atherosclerotic disease and shortest telomeres may have been too ill to participate, and this could have led to a specific survival effect in the studied population. Moreover, it is possible that more of these patients are receiving CVD-protective drugs (such as statins or aspirin) which could have an indirect impact on telomere length.

Regarding telomerase activity, it generally seems increased in SLE patients as compared to controls (Kurosaka et al. 2003; Kurosaka et al. 2006; Klapper et al. 2004; Kurosaka et al. 2001; Katayama & Kohriyama 2001). Unfortunately, this up-regulated telomerase activity is not capable of counteracting accelerated telomere attrition, resulting in shorter telomeres in SLE patients, thus possibly contributing to an accelerated senescence phenomenon.

### 2.2.2 Metabolism

Aging is characterized by a progressive deterioration in the maintenance of metabolic homeostatic processes (Barzilai et al. 2012). Particularly, a decline in the nutrient-sensing pathways is a key feature of aging and is the focus of intense investigation. The insulin-like growth factor 1 (IGF-1) and insulin signaling, and the mechanistic target of rapamycin (mTOR) pathways are major contributors to the aging process, highly conserved through evolution. mTOR is a serine/threonine kinase belonging to the highly conserved phosphoinositide 3-kinase (PI3K)-related kinase family, composed of two complexes, mTOR complex 1 (mTORC1) and mTORC2. The mTOR signaling pathway senses and integrates a variety of environmental cues in order to regulate the growth and the metabolism of cells. This pathway has been implicated in an increasing number of age-related pathological conditions, including cancer, obesity, type 2 diabetes or neurodegeneration (Laplante & Sabatini 2012). It is, as well, a critical mediator of mammalian longevity, as intense trophic and anabolic activities are important accelerators of aging whereas many of the mutations that extend lifespan decrease the activity of nutrient-signaling pathways (Fontana et al. 2010).

Human aging is also associated with a progressive mitochondrial dysfunction, which plays a complex role in regulating longevity (Wang & Hekimi 2015). Defective mitochondrial function is associated with defects in mitochondrial integrity, reduced mitochondrial biogenesis, and with a decrease in the efficacy of the respiratory chain and an increased reactive oxygen species (ROS) production. Imbalance between ROS production and antioxidant defenses can participate to increased oxidative stress, highly detrimental (Liguori et al. 2018).

In SLE, a lot of research has been carried out on the metabolic determinants of the disease pathogenesis. Studies in murine models as well as in lupus patients have clearly established that immune cells and especially T lymphocytes have profound rearrangements of

several metabolic pathways. They have an hypermetabolic state characterized by high glucose flux, oxidation, mitochondrial abnormalities and increase in mTOR kinase activities (Reviewed in (Morel 2017; Teng et al. 2020)). mTOR pathway participates in the proliferation of immune cells and has a fundamental role in T cell lineage commitment. Thus, mTOR signaling pathway promotes the differentiation of the pro-inflammatory Th1 and Th17 cells subsets and is central in the differentiation of T follicular helper (Tfh) cells, which are all critical to the pathogenesis of the disease (Zeng et al. 2016; Delgoffe et al. 2009). mTOR signaling pathway also affects the secretion of inflammatory cytokines (Lai et al. 2013) and the increased mTORC1 activity observed in SLE patients has been associated with a contraction of the T regulatory (Treg) cells compartment (Kato & Perl 2014; Kato & Perl 2018).

SLE T cells also depict mitochondrial dysfunction and increased oxidative stress. T cells mitochondria are characterized by a large size, membrane hyperpolarization, enhanced activity with increased production of ROS and ATP depletion (Doherty et al. 2014; Yin et al. 2015; Gergely et al. 2002). In one study, increased oxidative stress appeared to be independent of disease activity levels (Avalos et al. 2007).

Targeting metabolic pathways with specific interventions have shown promising effects in slowing down the aging process and is an area of intense research (Longo et al. 2015). One intervention that has proven to be by far the most effective in delaying or preventing an important range of age-related processes and extending the maximum lifespan in a wide range of organisms is caloric restriction (CR), *i.e.* the reduction of caloric intake (by 10% to 40%) without causing malnutrition (Liang et al. 2018). CR is an intervention likely to be difficult to implement in the long run in humans and pharmacological interventions have be proposed. Thus, inhibition of the mTOR signaling pathway with rapamycin significantly extends lifespan in model organisms (such as invertebrates and mice) and confers protection against several age-

related diseases (Kapahi et al. 2010; Harrison et al. 2009; Johnson et al. 2013). Additionally, metformin has emerged as a powerful antiaging drug (Barzilai et al. 2016): it increases lifespan of nematodes and mice (Anisimov et al. 2011; Cabreiro et al. 2013; Onken & Driscoll 2010) and is associated with reduction of age-associated pathologies such as dementia (Campbell et al. 2018).

Targeting these pathways have also shown promising effects in SLE. CR appeared to be protective in murine models of SLE. It decreases serum levels of anti-dsDNA antibodies, attenuates lupus nephritis, decreases pro-inflammatory cytokines expression and increases mice survival (Sun et al. 2004; Troyer et al. 1997; Muthukumar et al. 2000; Fernandes et al. 1976; Fernandes et al. 1978). In mouse models of the disease, antioxidants and rapamycin treatment seem to ameliorate lupus nephritis and to markedly prolong the animals survival (Warner et al. 1994; Gu et al. 2016; Suwannaroj et al. 2001). In humans, reducing mTOR activation with rapamycin or treatments with antioxidant effects (such as N-acetylcysteine) appeared to reduce disease severity in small groups of selected patients (Lai et al. 2012; Lai et al. 2018; Fernandez et al. 2006). Finally, oral administration of metformin in sanroque mice (Roquin<sup>san/san</sup>) normalizes T cell responses and suppresses the development of autoantibody-producing plasma cells (Lee et al. 2017). Metformin, delivered as an add-on treatment to standard of care, appears to improve disease parameters and reduce disease flares in patients with SLE (Wang et al. 2015; Sun et al. 2020).

Activation of mTOR pathway and oxidative stress appear to be substantial contributors to disease pathogenesis and to organ damage in SLE patients (Perl 2013). Targeting specifically these pathways seems to have a therapeutic benefit in murine models and in lupus patients. To our knowledge, the impact of these treatments was mainly focused on disease activity and

features of autoimmunity. No data seems available regarding the eventual impact of these interventions on co-morbidities and age-related diseases in this specific population.

### 2.2.3 Stem cells

Aging is associated with a decline in stem cell function leading to a loss of regenerative potential of tissues (Schultz & Sinclair 2016; Ahmed et al. 2017). This declining function affects all adult stem cell compartments: neural (NSCs), mesenchymal, adult skeletal muscle (also known as satellite cells) and hematopoietic stem cells (HSCs). Stem cell exhaustion is believed to be the result of the combination of surrounding environmental changes, and other hallmarks of aging described in the other sections, including increase in DNA damage, telomere shortening, mitochondrial dysfunction, metabolic changes and epigenetic patterns modifications.

In SLE, research has been mainly focused on bone marrow mesenchymal stem cells (BM-MSCs). These cells are bone-marrow-derived non-hematopoietic cells of mesodermal origin, characterized by self-renewal and pluripotent differentiation capability. They provide a supportive microenvironment for the maintenance of HSCs and for hematopoiesis. They also possess the ability to differentiate into various cell types, such as bone, fat or cartilage, under appropriate environmental conditions. Multiple papers have demonstrated that BM-MSCs from SLE patients are clearly defective. As compared to matched controls, lupus BM-MSCs exhibit an accelerated senescent phenotype, with lower proliferation capacity, flattened morphology and cytoskeleton abnormalities (Sun et al. 2007; Gu et al. 2014; Gao et al. 2017; Li et al. 2012; Nie et al. 2010; Gu et al. 2012; Gu et al. 2014). This senescent phenotype was observed in both untreated and treated SLE patients (Gu et al. 2012). BM-MSCs are more prone to undergo apoptosis, they exhibit increased DNA damage, overactivation of the mTOR pathway, increased intracellular ROS levels and increased production of proinflammatory cytokines,

similar to a senescent-associated secreted phenotype (Gao et al. 2017; Li et al. 2012; Gu et al. 2013; Gu et al. 2016).

To our knowledge, very limited data is available on the other stem cell compartments in SLE. Regarding HSCs, their function was investigated in one study on a lupus mouse model (Niu et al. 2013). Animals with active disease have an expanded HSCs population in the bone marrow and are characterized by a biased hematopoietic output toward the myeloid rather than the lymphoid lineage, driven by both genetic factors and chronic inflammation (Niu et al. 2013). Here, there was no investigation on the presence of an eventual accelerated senescence in HSCs.

Conclusively, there is evidence that SLE BM-MSCs display an enhanced aging process. Unfortunately, to our knowledge, the exact significance of this accelerated cellular senescence, from a clinical point of view on SLE disease activity and on the development of other comorbidities, was not clearly investigated.

#### 2.2.4 Proteostasis

Protein homeostasis, or proteostasis, corresponds to the collection of cellular mechanisms involved in maintaining the homeostasis of the proteome. Proteostasis is maintained through the coordinated action of several quality control mechanisms. The main components of the proteostasis maintenance system are chaperones, and two proteolytic systems, the ubiquitin-proteasome system and the lysosomal pathways (autophagy). All together, they coordinate protein synthesis with correct folding, conservation of protein conformation and protein degradation. Many studies have demonstrated that proteostasis is altered with age (Hipp et al. 2019; Kaushik & Cuervo 2015). Accumulation of external and endogenous stresses lead to the decline of the proteostasis network capacity and to the accumulation of misfolded and aggregated proteins. More stable proteomes have been observed in the longest-living species (Treaster et al. 2014; Pérez et al. 2009), whereas accumulation and

aggregation of misfolded proteins are found in common age-associated diseases, especially in neurodegenerative ones (*i.e.*, Alzheimer's and Parkinson's diseases) (Hipp et al. 2019; Kaushik & Cuervo 2015).

To our knowledge, few studies have investigated protein homeostasis during SLE. Some reports have been made on the implication of Heat Shock proteins (HSPs) in autoimmune diseases. HSPs are small molecular chaperones which interact and assist in the folding and assembly of the native polypeptide chains into its final structure. HSPs are expressed constitutively and in response to stressful conditions. Elevated expression of the 90 kDa HSP (HSP90) has been found in serum and kidneys of SLE patients (Kenderov et al. 2002; Shimp et al. 2012; Ripley et al. 2001; Hu et al. 2006). The SLE patients with active disease exhibit higher HSP90 levels than patients with inactive lupus (Hu et al. 2006). Positive correlations were found between HSP90 levels and IL-6 levels in SLE patients (Hu et al. 2006; Ripley et al. 2001). However, to our knowledge, no study has reported the presence of misfolded proteins or the evidence of a deficiency in the proteostasis network capacity in SLE under resting or stressful conditions.

### 2.2.5 Adaptation to stress

All along their lifetime, organisms have to face multiple stressors, which can be external perturbations, such as exposure to radiations, changes in oxygen levels, in temperature or in nutrient availability, as well as internal perturbations, such as protein or DNA damage. In response, cellular and organismal homeostasis are maintained through the coordinated action of multiples stress responses and repair pathways. During aging, there is progressive decline in all these critical response systems, as we have seen in the previous sections (IGF signaling, mTOR pathway, mitochondrial function, DNA damage response and proteostasis especially), leading to a deterioration of stress resistance (Haigis & Yankner 2010). In higher levels

organisms such as humans, stressors are not just physical, but are also social and psychological, adding another layer of complexity to the concept of stress and stress response. The impact of these specific stressors and their associated mechanisms on the aging process and aging rate is far to be completely understood currently (Epel & Lithgow 2014).

If we consider stress in the context of SLE, research has been mainly focused on the impact of psychological stressors on lupus occurrence and activity. For example, exposure to trauma and post-traumatic stress disorder have been associated with increased risk of developing SLE (O'Donovan et al. 2015; Feldman et al. 2019; Roberts et al. 2017). Furthermore, stress can have an impact on patients' functionality, as it is an important contributor to fatigue and as it can worsen the perceived clinical symptomatology (Williams et al. 2014; Azizoddin et al. 2019; Peralta-Ramírez et al. 2004). However, to our knowledge, there has been little specific investigation on the systems biology of stress response and its possible implication in the occurrence of age-related comorbidities in SLE.

#### 2.2.6 Inflammation and immunosenescence

Aging is characterized by a chronic, sterile, low-grade inflammation, called inflammaging (Franceschi et al. 2000), which results from an imbalance between inflammatory and anti-inflammatory networks (Franceschi et al. 2007). It is characterized by increased basal levels of circulating pro-inflammatory factors, such as C-reactive protein (CRP), IL-6, IL-1 or TNF- $\alpha$ , as well as chronic immune activation. Inflammaging contributes greatly to the pathogenesis of multiple chronic diseases and has been associated with higher risk of mortality (Franceschi & Campisi 2014). It results from multiple causes, such as danger signals which can be exogenous (persistent viral infections such as cytomegalovirus, failure to effectively clear pathogens) or endogenous (accumulation of damaged and senescent cells, cell debris, extracellular mitochondrial DNA).

Inflammaging is tightly linked to immunosenescence. Interestingly, it is known that a chronic immune activation, such as seen in autoimmune diseases, can accelerate the immunosenescence process. Thus, different autoimmune diseases, such as rheumatoid arthritis, have been associated with phenotypic changes that can be interpreted as an accelerated immunosenescence (Bauer 2020). This appears to be also the case in SLE patients who present elevated markers of immunosenescence. For example, as compared to age-matched healthy controls, they have expanded effector T cells subpopulations that have lost the expression of CD28, a marker that has been associated with immune aging (Kalim et al. 2020; Thewissen et al. 2007; Ulfah et al. 2019). SLE patients present an accumulation of terminally differentiated memory T cells, which harbor a decreased proliferative capacity and an increased tendency to undergo apoptosis (Fritsch et al. 2006). Interestingly, the immune-senescent profile observed in SLE patients has been associated with cognitive impairment (Kalim et al. 2020).

One believed key parameter of immunosenescence is the thymus, as it undergoes major changes during aging with a process of involution and shrinking, and a constant decline in the output of newly generated T lymphocytes (Thomas et al. 2020). Thymus functional capacity can be estimated by the frequency of peripheral cells expressing T-cell receptor excision circles (TRECs). In SLE patients as compared to age-matched healthy controls, it was observed a significant decrease in the number of TRECs in peripheral blood (Thewissen et al. 2007; Kayser et al. 2004; Fasching et al. 2017). Interestingly, the decrease in naïve CD4<sup>+</sup> T cells assessed with TRECs was found in older SLE patients, whereas no difference was observed for young patients and their respective controls, suggesting a disproportional decline in thymic output (Fasching et al. 2017).

### 2.2.7 Epigenetics

Epigenetics refers to the range of heritable reversible changes in chromatin structure and gene function that do not involve alterations of the DNA sequence itself. The major epigenetic mechanisms, *i.e.* DNA methylation, post-translational histone modifications and small non-coding RNAs, affect cells and tissues throughout life and play crucial roles in cellular differentiation, growth and development. Moreover, these three main categories of epigenetics marks are considered as central regulators of the aging process (Pal & Tyler 2016; Sen et al. 2016). Firstly, important changes in DNA methylation patterns have been observed during aging and have been associated with several age-related conditions. Different types of DNA methylation changes coexist during aging: the global genomic DNA methylation level decreases with age, mainly reflecting a decrease in DNA methylation of repetitive genomic regions and interspersed elements (Bollati et al. 2009; Luo et al. 2014); differential methylation of specific genomic loci occurs, especially with an age-related hypermethylation in gene promoters (Bell et al. 2012; Bell et al. 2016; Rakyan et al. 2010; Christensen et al. 2009); and there is an increase in the inter-individual divergence between patterns of DNA methylation (named DNA methylation drift) (Fraga et al. 2005; Maegawa et al. 2017; Tan et al. 2016; Mendelsohn & Larrick 2017), and an increase in the rate of epimutations (Gentilini et al. 2015). Secondly, histones, which are proteins that form an octamer core particle around which DNA is wrapped, undergo different types of posttranslational modifications (methylation, acetylation, ubiquitination, phosphorylation) that can induce changes in chromatin structure. Changes in the abundance and distribution of certain of these specific histone marks have been observed during aging in diverse species (Wang et al. 2018). Finally, noncoding RNAs, and especially microRNAs (miRNAs) which are a broad class of small noncoding RNAs that exert key roles in the post-transcriptional regulation of gene expression by targeting specific mRNAs,

are differentially expressed during aging (Huan et al. 2018; Noren Hooten et al. 2013; Smith-Vikos & Slack 2012; Inukai et al. 2012).

Contribution of epigenetic mechanisms to SLE pathogenesis has been extensively investigated (Reviewed in (Hedrich 2017; Long et al. 2016; Surace & Hedrich 2019)), especially regarding DNA methylation and identification of specific miRNAs signatures (Stagakis et al. 2011; Te et al. 2010; Dai et al. 2007; Tang et al. 2009; Chauhan et al. 2014). SLE is characterized by wide-spread DNA methylation changes. A lower overall DNA methylation level is observed in SLE subjects as compared to non-SLE individuals (Javierre et al. 2010). This global hypomethylation appears to be particularly important in repetitive regions and interspersed elements, derived from transposable elements, which form the majority of genomic DNA (long interspersed nuclear elements (LINEs), short interspersed nuclear elements (SINEs, such as Alu sequences), long-terminal repeat (LTR) transposons, or DNA transposons). Methylation levels of LINE-1 sequences and of Human Endogenous Retrovirus (HERV) sequences are decreased in SLE patients as compared to controls (Nakkuntod et al. 2011; Fali et al. 2014; Huang et al. 2014; Nakkuntod et al. 2013). Hypomethylation of these regions can increase the expression of their respective transcripts and can increase DNA immunogenicity, providing potent stimuli for an innate immune response (Crow 2010). Epigenetic mechanisms are also crucial in the dysregulation of the expression of genes pertinent to SLE pathogenesis. This influence of the expression of SLE-related genes is particularly true in T cells (Reviewed in (Zhang et al. 2013)), leading to imbalanced expression of crucial factors, such as CD40L, FoxP3, perforin, or several cytokines (IL-10, IL-17, IL-4, IL-6) (Hedrich et al. 2014; Zhao et al. 2010; Apostolidis et al. 2013; Lal et al. 2009, p.3; Kaplan et al. 2004). Epigenetic alterations are finally important contributors to the interferon signature in SLE. Thus, several studies on DNA methylation have reported striking patterns of hypomethylation

at interferon-signature genes, such as *IFI44L* (Interferon Induced Protein 44 Like), *MX1* (Interferon-Induced GTP-Binding Protein Mx1), *IFITM1* (Interferon Induced Transmembrane Protein 1), or *IRF7* (Interferon Regulatory Factor 7) (Imgenberg-Kreuz et al. 2018; Coit, Yalavarthi, et al. 2015; Yeung et al. 2017; Absher et al. 2013; Chen et al. 2019; Zhao et al. 2016; Coit, Renauer, et al. 2015; Imgenberg-Kreuz et al. 2019; Zhu et al. 2016; Coit et al. 2013).

Epigenetic modifications are highly complex and have appeared to be crucial contributors to SLE pathogenesis. To our knowledge, the contribution of these changes to SLE co-morbidities has not been investigated so far. Aside from lower DNA methylation levels in repetitive sequences which are also observed during the aging process, it does not seem that there is a significant overlap between SLE epigenetic signatures and age-related ones.

#### 2.2.8 Impact of therapeutics

SLE is a chronic disease characterized by a fluctuating disease course. Organ-threatening/life-threatening manifestations of the disease require an intensive treatment with an initial period of high-intensity immunosuppressive therapy to control disease activity (Fanouriakis et al. 2020). Subsequently, therapeutic consolidation and prevention of relapses/flares are based on long-term administration of specific therapeutics, such as hydroxychloroquine or glucocorticoids (GCs), which can be prescribed for many years (Mathian et al. 2020).

Some therapeutics used in SLE are known to have severe side effects, and they could have a negative impact on the aging process by favoring its acceleration. For example, high cumulative GCs dosage has been associated with atherosclerosis in patients with SLE (Skaggs et al. 2012), as GCs can have deleterious effects on insulin resistance, blood pressure and lipid profile. Additionally, it has been well demonstrated that cancer therapeutics can cause accelerated aging (Hill et al. 2020; Cupit-Link et al. 2017). In SLE, it is thus possible that exposure to intense

immunosuppressive therapy, with cyclophosphamide for example, may accelerate mechanisms associated with aging, such as precipitation of genomic instability.

Nevertheless, it should also be considered the fact that these therapeutics may have a positive impact of the aging process and its associated diseases. As mentioned earlier, it is clearly established that chronic inflammation plays a pathogenic role in the development of accelerated atherosclerosis, and therapeutic suppression of inflammation can have a favorable impact on CVD. Thus, anti-inflammatory strategies have been proposed for secondary prevention of atherosclerotic disease in the general population (therapeutic inhibition of interleukin-1 $\beta$  with canakinumab or treatment with methotrexate) (Ridker et al. 2017; Ridker et al. 2019). In the context of autoimmunity, it has been particularly investigated in rheumatoid arthritis. Lowering disease activity with the use of disease-modifying antirheumatic drugs (DMARDs) (methotrexate in particular) and/or biological agents (such as anti-TNF $\alpha$  agents) decreases CVD risk (Jagpal & Navarro-Millán 2018). In mice, B cell depletion therapy reduces the development of atherosclerosis (Ait-Oufella et al. 2010; Kyaw et al. 2010). In patients with SLE, hydroxychloroquine has been associated with decreased aortic stiffness (Selzer et al. 2001) and reduced carotid plaque burden (Roman et al. 2003). The anti-inflammatory effects of GCs, when used in a specific therapeutic window with low dose, in addition of preventing flares (Mathian et al. 2020), may be atheroprotective and may have beneficial impact on CVD risk.

While the clinical impact of the SLE therapeutics has been examined, especially regarding CVD risk, their exact influence on the basic cellular and molecular mechanisms of aging should be better investigated. Influence of medications on DNA damage and telomere length has been explored in some reports. In three different studies, no correlations have been found between genomic instability and SLE treatment modalities (Singh et al. 2014; Souliotis & Sfikakis 2015;

López-López et al. 2014). Influence of GCs treatment on telomere length and on telomerase activity is disputed (Wu et al. 2007; Haque et al. 2013; Kurosaka et al. 2001; Kurosaka et al. 2003). Regarding stem cells, the senescent phenotype of BM-MSCs was observed in both untreated and treated SLE patients (Gu et al. 2012). To our knowledge, the exact impact of SLE medications on the other pillars of aging has been poorly investigated. Their beneficial or detrimental effects, difficult to capture owing to several confounding factors such as disease activity and disease duration, require longitudinal studies in SLE patients cohorts in order to clarify this issue.

### 2.2.9 Conclusion

SLE pathogenesis is extremely complex, involving genetic, environmental, hormonal and immunological factors (Tsokos et al. 2016). Multiple pathways involved in this pathogenesis appear to have a complex interplay with the ones which underpin the aging process (Kennedy et al. 2014; López-Otín et al. 2013). Some of the biological hallmarks that have been proposed for aging can be identified chronologically earlier in subjects with SLE, and typical age-associated mechanisms, such as DNA damage or telomere attrition, are frequently found in SLE patients at young age. Of particular importance is the evidence of the presence of an acceleration of aging in the immune system during SLE. The exact relevance and implications of these observations on SLE disease course on one hand, but mostly on the presence of a clinically significant acceleration of the aging process at the systemic level on the other hand is not clear and several questions remain to be answered. Longitudinal studies with frequent serial measurements of aging biological hallmarks, evaluation of the occurrence of age-related diseases and evaluation of the impact of therapeutics are greatly needed.

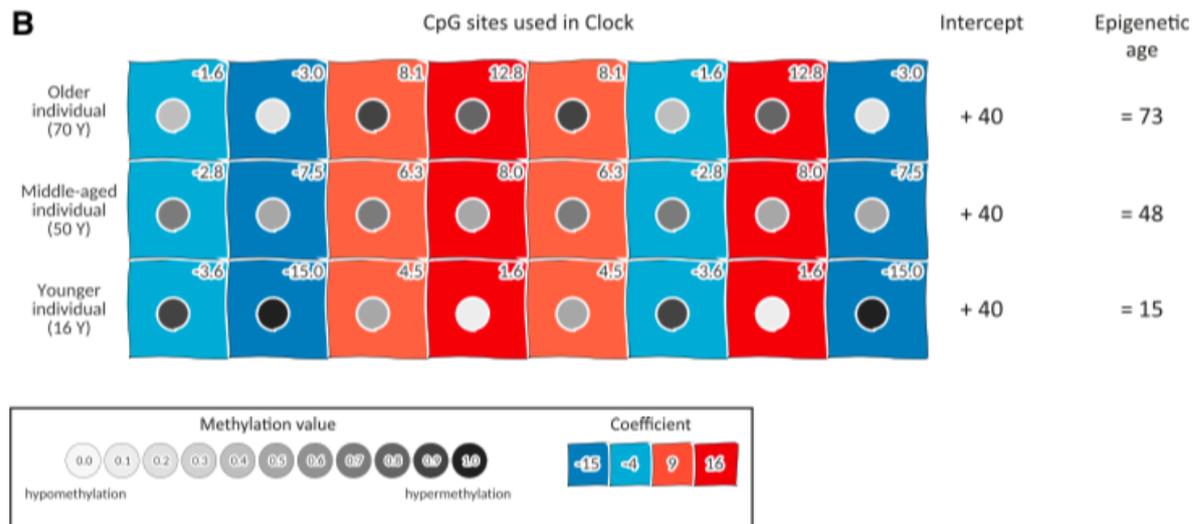
### ***3. SLE and epigenetic biomarkers of age: a pilot study***

#### ***3.1 Epigenetic biomarkers of aging: definition and concept***

In order to evaluate if a condition is associated or not with an acceleration of the aging process, we need to find a way to properly measure biological aging. To this aim, identification of biomarkers of aging has been a critical and intense area of research since decades. Ideally, these markers could be used to predict the occurrence of adverse outcomes during aging and to identify populations with accelerated aging which could benefit from early interventions. Biomarkers of age could also be used as surrogate endpoints for clinical trials and to evaluate the effectiveness of an intervention.

There is currently no consensus on how to best measure one individual's biological age and until now a valid and unique aging biomarker has not yet been identified. However, in the last decade, there has been tremendous interest in epigenetic biomarkers of aging (also known as epigenetic clocks or DNA methylation-based biomarkers). Epigenetic clocks are algorithms based on the combination of DNA methylation levels at some specific CpG sites, that can predict the age of a DNA source. They are nowadays considered as among the most robust predictors of chronological age in humans (Horvath & Raj 2018; Field et al. 2018; Jylhävä et al. 2017; BLUEPRINT consortium 2016). Two of these clocks, Horvath's (based on 353 CpG sites) and Hannum's (based on 71 CpG sites) calculators, developed in large samples cohorts covering the entire adult lifespan, show high correlations with age and small deviations from chronological age (Horvath 2013; Hannum et al. 2013). Besides these two models, researchers have also developed several other age-prediction models with different characteristics and performances (Levine et al. 2018; Lu et al. 2019; Horvath et al. 2018; Bocklandt et al. 2011; Weidner et al. 2014). These clocks are mostly derived by linear regression algorithms that train against the chronological age of the sample and ultimately select a set of CpG sites. The weights associated with each CpG site are determined and the product of the methylation level at each

site with the learned coefficient is summed, leading to an estimated epigenetic age, also called DNAm age (**Figure 2**).



**Figure 2:** DNA methylation clock: how it works. Adapted from Field *et al.* 2018.

Epigenetic clocks are not only able to predict chronological age with high accuracy, but evidence also suggests that they can be informative of the biological age of an individual. Thus, a positive or negative deviation of the biological age to chronological age according to these models reflects the presence of an accelerated or decelerated aging phenomenon, respectively. Horvath's and Hannum's clocks are able to predict all-cause mortality independently of classic risk factors (Marioni *et al.* 2015; Chen *et al.* 2016; Dugué *et al.* 2018; Zheng *et al.* 2016; Christiansen *et al.* 2016; Perna *et al.* 2016; Fransquet *et al.* 2019; Marioni *et al.* 2016) and they also can be informative on other age-related outcomes such as frailty, cognitive and physical functioning, neurodegenerative or cardiovascular diseases (Horvath & Raj 2018). Finally, it was also demonstrated that Horvath's clock is able to capture the age acceleration phenomenon associated with progeroid syndromes, such as Down or Werner syndromes (Maierhofer *et al.* 2017; Horvath, Garagnani, *et al.* 2015) and to detect age-deceleration effects in models of

healthy aging and longevity, such as centenarians and their offspring (Horvath, Pirazzini, et al. 2015). Interestingly, epigenetic clocks have also been applied to cohorts of patients with chronic HIV infection, which is associated with early onset of diseases linked to aging (Wing 2016). According to two studies, HIV-infected treated patients are characterized by a 5 year increase in biological age in blood samples as compared to healthy controls, reflecting an acceleration of the aging process from a biological point of view (Gross et al. 2016; Horvath & Levine 2015).

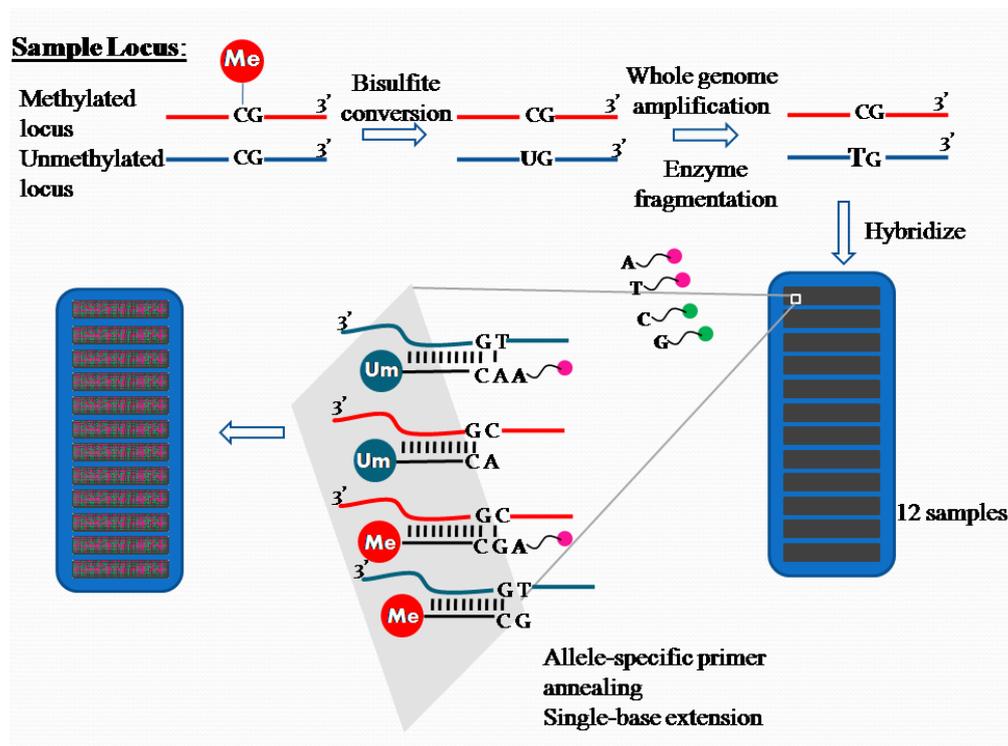
In the current study, we sought to test the hypothesis that SLE would be associated with an accelerated biological aging. The objective of this work was to assess the association between lupus status and epigenetic age acceleration, by comparing DNA methylation profiles of SLE patients *versus* healthy controls.

### **3.2 Methods**

Publicly available DNA methylation datasets involving SLE patients were searched on NCBI's Gene Expression Omnibus (GEO) database ([www.ncbi.nlm.nih.gov/gds](http://www.ncbi.nlm.nih.gov/gds)). The database was searched for DNA methylation data generated with the genome-wide Illumina<sup>®</sup> Infinium platforms (Illumina Inc., San Diego, CA, USA). All generations of platforms were considered: 27K (GEO Accession: GPL8490), 450K (GEO Accession: GPL13534) and EPIC (GEO Accession: GPL21145). The Illumina<sup>®</sup> Infinium HumanMethylation27 BeadChip array interrogates 27 578 methylation sites quantitatively across the genome at single-nucleotide resolution, while the HumanMethylation450 BeadChip array interrogates over 450 000 methylation sites and the EPIC one over 850 000.

With these arrays, DNA methylation levels at individual CpG sites are evaluated following the same protocol. Firstly, genomic DNA is extracted from the tissue/cell type of

interest. Subsequently, 500 ng or 1 mg of DNA are bisulfite converted, using EZ DNA Methylation Kits from Zymo Research Corporation (Zymo, Orange, CA, USA). After bisulfite conversion, DNA is whole genome amplified, enzymatically fragmented and hybridized to the Illumina® Infinium BeadChips (Illumina Inc., CA, USA), according to manufacturer's protocols. Finally, arrays are scanned using specific instruments and DNA methylation data are extracted for processing and normalization (**Figure 3**).



**Figure 3:** Illumina® Infinium HumanMethylation 27K workflow (source: Wikimedia commons).

For this study, pre-processed DNA methylation data were directly downloaded from GEO database. Information regarding studied samples, when available, was also retrieved: tissue/cell type used, socio-demographic factors of the participants and status (lupus patient / control).

DNA methylation data were then uploaded into the DNA methylation age calculator, developed by Steve Horvath and freely available at the website: <https://dnamage.genetics.ucla.edu>, in order to calculate the epigenetic age of the individuals,

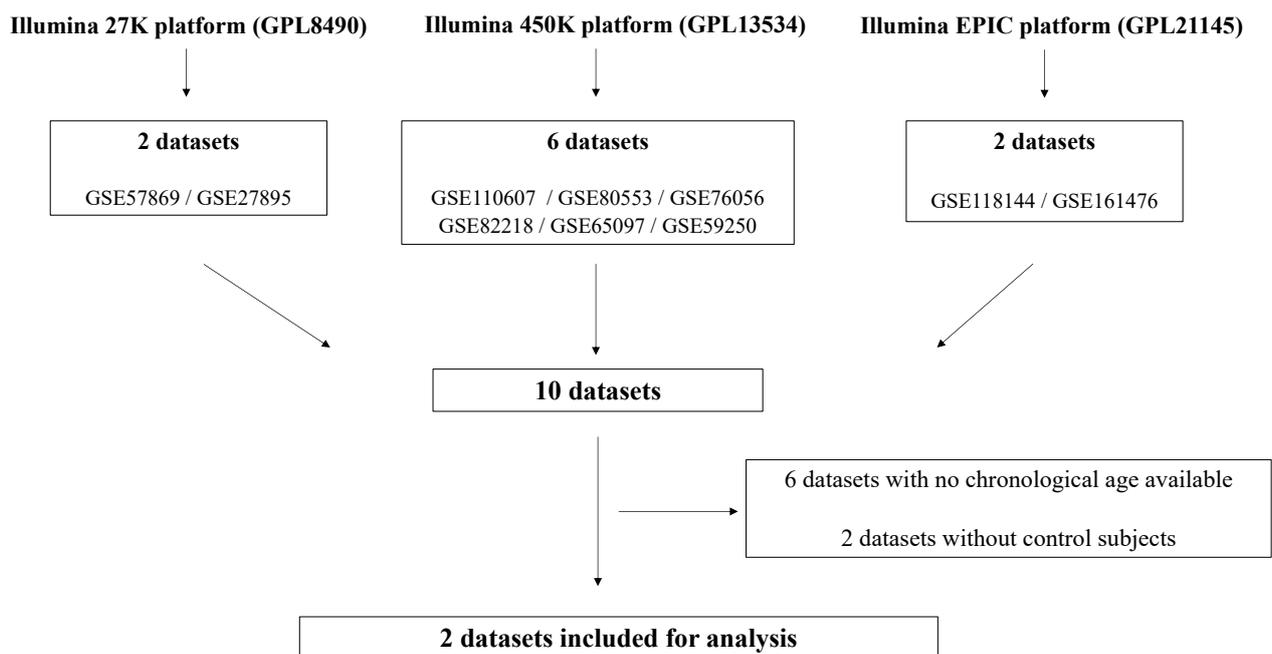
named DNAm age. DNAm age is calculated using the weighted average of DNA methylation levels at 353 CpG sites, as described in the seminal paper (Horvath 2013). We also considered a measure of epigenetic age acceleration, denoted AgeAccel, which corresponds to the residual that results from regressing DNAm age on chronological age and denoted AgeAccelerationResidual in the online calculator. A positive (or negative) value indicates that the biological age of the sample is higher (or lower) than expected based on chronological age.

All values are expressed as mean  $\pm$  standard deviation or median  $\pm$  interquartile range for continuous variables, and as counts and percentages for categorical variables. Pearson correlation coefficient was calculated to describe the linear correlation between measures of epigenetic age and chronological age. Wilcoxon tests were used to compare measures of epigenetic age acceleration between the different groups. Statistical significance was defined as p-value  $< 0.05$ . All statistical analyses and figures were made using R v3.3.2.

### 3.3 Results

#### 3.3.1 Datasets

Ten publicly available datasets involving SLE patients were found on GEO database. Six of them were excluded as they did not contain the chronological age of the included subjects, making the analysis impossible (GSE110607, GSE76056, GSE59250, GSE57869, GSE27895, GSE118144). Additionally, two datasets did not include control subjects (GSE80553 and GSE161476), making subsequent comparison impossible. In the end, a total of two datasets, GSE65097 and GSE82218, were selected for further analysis (**Figure 4** and **Table 1**).



**Figure 4:** GEO datasets selection process.

<b>GEO ID</b>	<b>Citation</b>	<b>Tissue source</b>	<b>Platform</b>	<b>N (total)</b>	<b>N SLE subjects</b>	<b>Female (%)</b>	<b>Mean age (years)</b>
GSE65097	(Coit, Yalavarthi, et al. 2015)	Neutrophils	450K	30	15	100	37.8
GSE82218	(Zhu et al. 2016)	PBMC	450K	55	30	84	30.5

**Table 1:** Overview of the DNA methylation datasets included in the analysis.

PBMC: Peripheral Blood Mononuclear Cells.

### 3.3.2 Lupus patients and controls

#### *GSE65097*

The GSE65097 dataset was generated by Coit *et al.* (Coit, Yalavarthi, et al. 2015). It consisted of 15 lupus patients and 15 age, sex and ethnicity matched healthy controls. Participants were recruited from the University of Michigan rheumatology clinics or from the Lupus Natural History Protocol at the National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS) of the National Institutes of Health (NIH). Mean age of patients and controls was 36.9 years ( $\pm 10.2$ ) and 38.7 years ( $\pm 9.6$ ), respectively. All study participants were female. Main characteristics of the lupus patients are presented in **Table 2**.

<b>Age</b> (mean ± SD)	36.9 years (± 10.2)
<b>Ethnicity</b> (n, %)	European-American: 6 (40%) African American: 5 (33%) Hispanic/White: 2 (13%) Asian: 2 (13%)
<b>SLEDAI score</b> (mean ± SD)	1.67 (± 1.84)
<b>Clinical manifestations</b> (n, %)	Cutaneous: 10 (67%) Articular: 10 (67%) Renal: 6 (40%) Hematologic: 8 (53%) Serositis: 7 (47%) Neurologic: 2 (13%)
<b>Treatments</b> (n, %)	Hydroxychloroquine: 10 (67%) Steroids: 11 (73%) Mycophenolate: 6 (40%) Azathioprine: 2 (13%) Rituximab: 1 (7%)

**Table 2:** Demographic and clinical information of the lupus patients included in the GSE65097 dataset.

SD: Standard Deviation. SLEDAI: Systemic Lupus Erythematosus Disease Activity Index.

In this study, neutrophils were extracted from the granulocyte layer after Ficoll density gradient centrifugation of fresh peripheral blood samples (25 mL). DNA methylation profiling was performed using the Infinium HumanMethylation450 BeadChip Kit.

In the published paper, authors identified 293 differentially methylated CpG sites in neutrophils between SLE patients and controls, the majority of them (68%) being hypomethylated. A robust and consistent hypomethylation of interferon signature genes in neutrophils of SLE patients was observed (Coit, Yalavarthi, et al. 2015).

#### *GSE82218*

The GSE82218 dataset was generated by Zhu *et al.* (Zhu et al. 2016). Thirty SLE patients (15 SLE with lupus nephritis (LN+) and 15 SLE without lupus nephritis (LN-)) and 25 controls were included in this study. Patients and controls were matched for age, sex, and ethnicity. They were all recruited from Xiangya Hospital, Central South University of China (Changsha, Hunan, China). Mean age of SLE LN+ patients, SLE LN- patients and controls was 30.13 ( $\pm$  7.68), 28.2 ( $\pm$  6.7) and 32.2 years ( $\pm$  9.04) respectively. Main characteristics of the lupus patients are presented in **Table 3**.

	SLE LN-	SLE LN+
<b>Age</b> (mean ± SEM)	28.2 years (± 6.7)	30.13 years (±7.68)
<b>Sex</b> (M/F)	2/13	2/13
<b>Disease duration</b> (months) (mean ± SEM)	4.90 ± 4.18	3.33 ± 4.21
<b>SLEDAI score</b> (mean ± SEM)	9.06 (± 4.06)	14.93 (± 4.65)
<b>Clinical manifestations</b> (n, %)	Malar rash: 8 (53%) Discoid rash: 1 (7%) Photosensitivity: 4 (27%) Oral ulcers: 2 (13%) Arthritis: 8 (53%) Serositis: 1 (7%) Renal: 0 (0%) Neurologic: 1 (7%) Hematologic: 2 (13%)	Malar rash: 11 (73%) Discoid rash: 3 (20%) Photosensitivity: 3 (20%) Oral ulcers: 1 (7%) Arthritis: 6 (40%) Serositis: 3 (20%) Renal: 15 (100%) Neurologic: 0 (0%) Hematologic: 7 (47%)

**Table 3:** Demographic and clinical information of the lupus patients included in the GSE82218 dataset.

SEM: Standard Error of the Mean. SLEDAI: Systemic Lupus Erythematosus Disease Activity Index. SLE LN-: Systemic Lupus Erythematosus without lupus nephritis. SLE LN+: Systemic Lupus Erythematosus with lupus nephritis.

SLE LN+ was defined as clinical and laboratory manifestations that meet American College of Rheumatology (ACR) criteria (persistent proteinuria > 0.5 g/day or greater than 3+ by dipstick, and/or urine cellular casts including red blood cells, hemoglobin, granular, tubular, or mixed). SLE LN+ patients exhibited higher disease activity (SLEDAI score) and lower level of

complement C3 ( $293.2 \pm 196.2$  versus  $534.5 \pm 280.1$  mg/dL) than SLE LN- patients. There was no other statistically significant difference between the two groups.

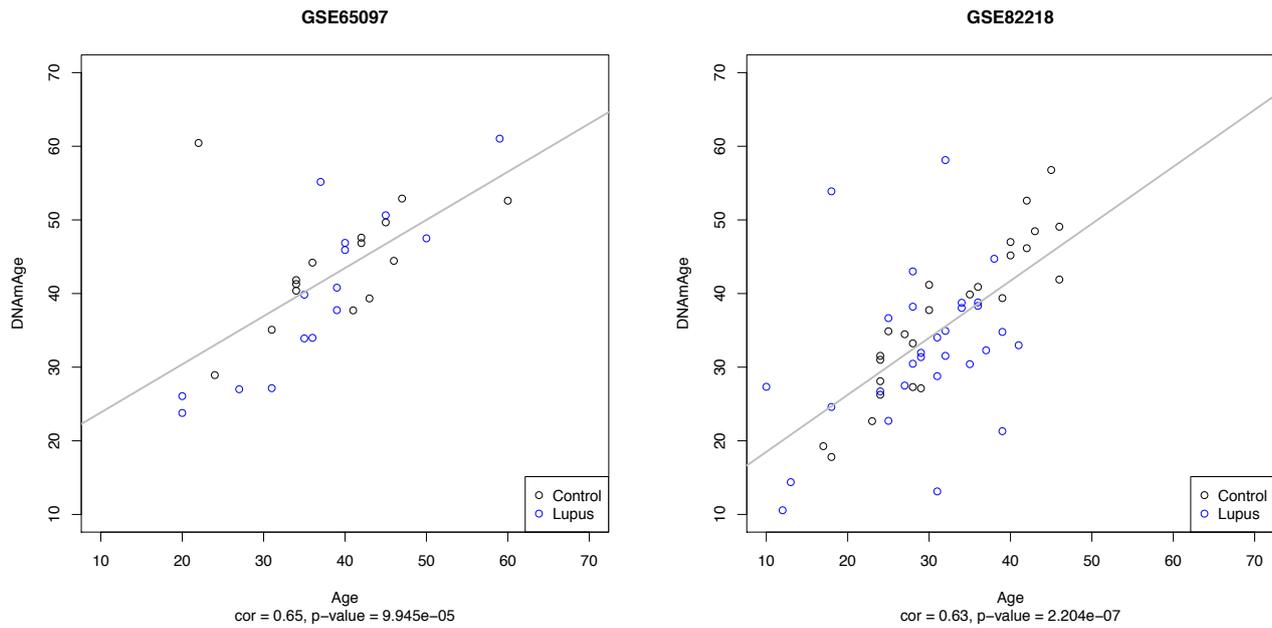
PBMC were isolated from heparinized blood by Ficoll density gradient centrifugation. DNA methylation profiling was performed using the Infinium HumanMethylation450 BeadChip Kit.

Here, the authors identified 1813 hypermethylated CpG sites and 3785 hypomethylated ones in SLE patients as compared to controls. Correlating the DNA methylation data with transcriptomic analysis, they demonstrated that DNA hypomethylation was related with overexpression of IFN-related genes (Zhu et al. 2016).

In both datasets, all patients studied fulfilled the ACR classification criteria for lupus (Tan et al. 1982). All patients and controls signed an informed consent prior to participation in the respective study, and protocols were approved by local ethics committees.

### 3.3.3 Epigenetic age

For each dataset individually, epigenetic age (also referred to as DNA methylation age (DNAm age)) was calculated using the online age calculator. As expected, DNAm age was significantly associated with chronological age ( $p < 0.0001$ ) in both cohorts (**Figure 5**).



**Figure 5: Significant association between DNAm age and chronological age in both cohorts.**

Scatter plots of chronological age (x-axis) versus DNAm age (y-axis) in the different groups (Control subjects = black; Lupus patients = blue). Grey lines represent fitted linear regressions.

### 3.3.4 Epigenetic age acceleration

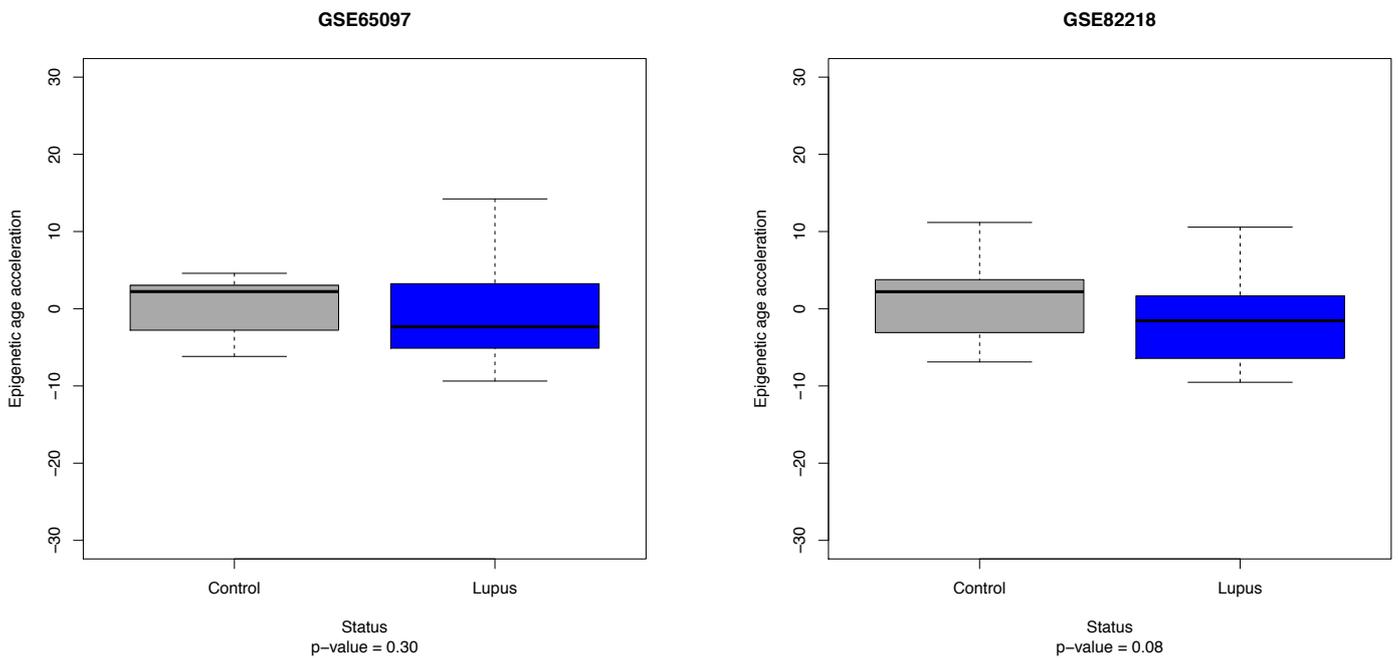
For each subject, we also considered the measure of epigenetic age acceleration, named AgeAccel, defined as the residual from regressing epigenetic age on chronological age. Positive values of epigenetic age acceleration indicate an epigenetic age older than expected, while negative values indicate an epigenetic age younger than expected on the basis of chronological age (**Table 4**).

<b>GEO ID</b>	<b>Status</b>	<b>Number of subjects</b>	<b>Chronological age (years)</b> mean $\pm$ SD	<b>DNAm age (years)</b> mean $\pm$ SD	<b>AgeAccel (years)</b> mean $\pm$ SD
<b>GSE65097</b>	Controls	15	38.7 $\pm$ 9.6	44.2 $\pm$ 7.9	2.0 $\pm$ 8.8
	SLE	15	36.9 $\pm$ 10.2	39.8 $\pm$ 11.3	- 1.0 $\pm$ 6.0
<b>GSE82218</b>	Controls	25	32.2 $\pm$ 9.0	36.8 $\pm$ 10.4	1.1 $\pm$ 4.9
	SLE	30	29.1 $\pm$ 8.1	32.3 $\pm$ 10.4	- 0.9 $\pm$ 10.2

**Table 4: DNAm age and measure of epigenetic age acceleration in the two cohorts.**

SD: standard deviation. DNAm age: DNA methylation age. AgeAccel: Epigenetic age acceleration.

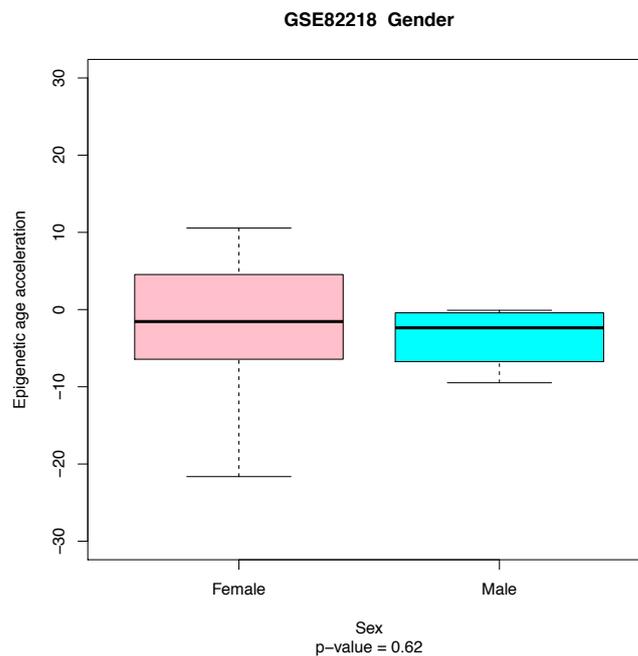
Subsequently, in both cohorts, we compared the average epigenetic age acceleration measure in controls and in lupus patients, in order to evaluate if lupus patients are biologically older than their age-matched controls. We did not observe any statistically significant difference between the groups (GSE65097: p-value = 0.30; GSE82218: p-value = 0.08) (**Figure 6**). In both datasets, lupus patients appeared to be slightly biologically younger as compared to the control subjects, but the trend was not statistically significant.



**Figure 6:** Boxplots of predicted epigenetic age acceleration in control subjects (grey) and in lupus patients (blue).

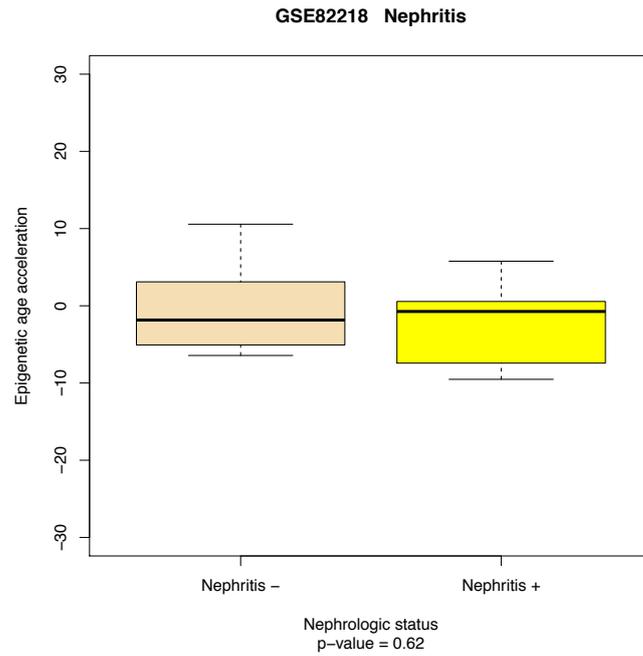
### 3.3.5 Influence of sex and lupus nephritis on the measure of epigenetic age acceleration

In GSE82218 dataset, males and females were included. We divided the cohort of lupus patients according to sex in order to evaluate the potential relationship between sex and the measure of epigenetic age acceleration in this specific population. There was no difference between the two groups (p-value = 0.62) (**Figure 7**). It should be noted that the number of male subjects is extremely low (n=4).



**Figure 7: Boxplot of predicted epigenetic age acceleration in SLE females (pink) and SLE males (blue).**

Finally, in this dataset, we also divided SLE patients according to the presence or the absence of lupus nephritis. Despite a trend toward a higher epigenetic age acceleration measure in patients with lupus nephritis (Mean AgeAccel = -0.5 years) as compared to subjects without nephritis (Mean AgeAccel = -1.3 years), the difference between the two groups was not statistically significant (p-value = 0.62) (**Figure 8**).



**Figure 8: Boxplot of predicted epigenetic age acceleration in SLE patients with lupus nephritis (yellow) and without lupus nephritis (beige).**

## 4. Discussion

SLE is a complex systemic autoimmune disease, with diverse clinical manifestations and an unpredictable clinical course. Despite improvements in diagnosis and treatments, many SLE patients can suffer from the accumulation of organ damage and mortality risks remain high for this population. Especially, clinical progression is associated with the development of comorbidities, such as infections or cardiovascular events, which are classically considered as age-related in the general population. Thus, the high prevalence of these comorbidities has raised the question of the presence of an acceleration of the aging process in the SLE population.

Interestingly, from a clinical point of view, two recent papers have evoked the importance of frailty in SLE (Katz et al. 2017; Legge et al. 2020). Frailty has been conceptualized as a state of increased vulnerability which results in a diminished ability to respond to physiologic stressors and in poorer outcomes. While the concept was so far mainly developed and applied in geriatrics communities, it has also been extended to other populations. In 2017, Katz *et al.* applied the classic Fried frailty criteria (Fried et al. 2001) in a cohort of 152 women with SLE (mean age = 48 ( $\pm$ 12) years) (Katz et al. 2017). Prevalence levels of frailty were found really high, as compared to the ones that can be observed in populations of older adults. Thus, 20% of the sample was classified as frail and 50.7% as prefrail. In this study, presence of frailty was associated with an increased risk of functional decline and mortality. More recently, using data from the Systemic Lupus International Collaborating Clinics (SLICC) inception cohort, Legge *et al.* developed a frailty index specially tailored for lupus patients, named SLICC-FI, based on the combination of 48 health deficits (Legge et al. 2020). Here also, high prevalence of frailty (27.1%) was found among SLE patients (mean age = 35.7 ( $\pm$ 13.4) years), higher than expected for similarly aged individuals of the general population.

From a biological point of view, as we have seen in the first part of this work, multiple pathways involved in the pathogenesis of SLE have a complex interplay with the ones which

underpin the aging process. Some of the typical biological hallmarks of aging, such as telomere attrition, DNA damage or stem cells decline, can be identified prematurely in SLE. Particularly, patients with SLE demonstrate several signatures of accelerated aging in the immune system.

In order to test the accelerated aging hypothesis in the SLE population, in the second part of the present work, we investigated the cross-sectional association between lupus status and epigenetic age acceleration in two cohorts of lupus patients. We did not find any evidence of an accelerated biological aging according to this measure of biological age. The results we obtained were consistent between the two independent datasets which used different cell types for the measure of DNA methylation. In the opposite direction from what would be expected, SLE patients appeared to be slightly biologically younger than controls, but the difference was not statistically significant. On the contrary, in one dataset, measures of epigenetic age acceleration in patients with SLE nephritis were higher than the ones measured in patients without nephritis, suggesting a discrete acceleration of the aging process in the first subgroup, but again the difference was not significant. While SLE patients appear to have an acceleration of the aging process according to other indices of aging such as telomere attrition, we did not find such an association with epigenetic biomarkers of age. Different hypothesis can be put forward to explain these null associations. It is possible that SLE patients do not have an acceleration of the aging process, or it is possible that it exists but is not captured by the model used in this work, and that DNAm age is not a suitable biomarker for age in SLE patients.

Our study has important limitations. Firstly, it relies on data of public repositories and highlights the problematic of the accessibility of data. While dozens of studies have been made on SLE and DNA methylation, and several of them used the Illumina® platform (more than 20 studies according to our research in PubMed), very few data are made publicly available. In the

end, we were able to include only two datasets. Moreover, these datasets are characterized by a cross-sectional design, small sample sizes and narrow age range. Data on disease activity, disease duration and therapeutics were presented in the original publication, but the information was not available individually in the downloaded data. We tried to reach corresponding authors of both publications (A.H Sawalha; X. Zuo and Q-Z. Li) by e-mail in order to obtain raw data, but we did not get a reply so far. As this individual information was not available to us, we were not able to assess the effect of these factors on DNAm age.

The two datasets included relatively young lupus patients, with relatively inactive disease and short disease duration. It would be interesting to perform analysis of biomarkers of age in cohorts of SLE patients with wider ranges of age, of disease activity indexes and of disease durations in order to evaluate the impact of these parameters on the biological age of the individuals. Moreover, it should be noted that inclusion of young patients, especially in the second dataset (GSE82218), with age between 10 and 20 years old, raises the problem of the performance of the mathematical model to properly predict the biological age of the younger individuals, as it is known that there is non-linear (logarithmic) pattern of the clock during teenage years (Horvath 2013).

As mentioned earlier, in the two datasets, DNA methylation analysis were performed using different cell types: isolated neutrophils (GSE65097) or PBMC (GSE82218). Considering that DNA methylation is cell type specific, it is known that differences in cell counts can impact the prediction of the model. Using Horvath's model, measures of epigenetic age acceleration can be adjusted for cellular heterogeneity (Horvath & Ritz 2015), but this correction is only possible for whole blood samples and thus this normalization could not be applied here. Furthermore, we could not apply the other widely used model of epigenetic clock, *i.e.* the Hannum's model (Hannum et al. 2013), as it only applies to whole blood samples.

We did not identify any significant overlap between the CpG sites found differentially methylated into the two original publications and the CpG sites included in Horvath's clock. Thus, in the study by Coit *et al.* (GSE65097), there was no common CpG sites between the 293 differentially methylated positions and the CpG sites included in the Horvath's clock. In the other study (GSE82218), 5 CpG sites were common. Four of them (cg21096399 associated with *MCAM* gene; cg17285325 associated with *ECGF1* gene; cg04121983 associated with *CASKIN2* gene; cg06810647) were found hypomethylated in SLE patients as compared to controls, whereas the other one (cg26297688 associated with *C12orf23* gene) was found hypermethylated. It seems unlikely that these five positions have a strong influence on the outcome of the biological age prediction. Indeed, DNAm age is calculated through averaging the information of 353 CpG sites scattered across the genome and in the final model, each CpG site has a limited weight in the algorithm. Therefore, changes in DNA methylation levels at these 5 CpG sites have possibly only a marginal impact on the final outcome.

The ability of properly measuring biological age can provide interesting opportunities to identify individuals with premature aging who are at increased risk for adverse outcomes. Early identification of these subjects can lead to preventative measures for co-morbidities and precocious interventions to try to slow down aging. These tools can also be used to better predict long-term outcomes in SLE patients. To date, such instruments are limited in SLE. While interesting results have been recently obtained with the development of frailty index (Legge *et al.* 2020), more work is required for biological markers. We need more data on the complex interplay between SLE pathogenesis and the aging process, as well as on the possible influence of environmental factors and SLE medications especially. There is a need of longitudinal, prospective studies following individuals over the course of their disease, in order to better

characterize features of aging in SLE and assess the dynamics of potential biomarkers of age in this population.

## 5. References

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## Serment d'Hippocrate

Au moment d'être admise à exercer la médecine, je promets et je jure d'être fidèle aux lois de l'honneur et de la probité.

Mon premier souci sera de rétablir, de préserver ou de promouvoir la santé dans tous ses éléments, physiques et mentaux, individuels et sociaux.

Je respecterai toutes les personnes, leur autonomie et leur volonté, sans aucune discrimination selon leur état ou leurs convictions. J'interviendrai pour les protéger si elles sont affaiblies, vulnérables ou menacées dans leur intégrité ou leur dignité. Même sous la contrainte, je ne ferai pas usage de mes connaissances contre les lois de l'humanité.

J'informerai les patients des décisions envisagées, de leurs raisons et de leurs conséquences.

Je ne tromperai jamais leur confiance et n'exploiterai pas le pouvoir hérité des circonstances pour forcer les consciences.

Je donnerai mes soins à l'indigent et à quiconque me les demandera.

Je ne me laisserai pas influencer par la soif du gain ou la recherche de la gloire.

Admise dans l'intimité des personnes, je tairai les secrets qui me seront confiés. Reçue à l'intérieur des maisons, je respecterai les secrets des foyers et ma conduite ne servira pas à corrompre les mœurs.

Je ferai tout pour soulager les souffrances. Je ne prolongerai pas abusivement les agonies. Je ne provoquerai jamais la mort délibérément.

Je préserverai l'indépendance nécessaire à l'accomplissement de ma mission. Je n'entreprendrai rien qui dépasse mes compétences.

Je les entretiendrai et les perfectionnerai pour assurer au mieux les services qui me seront demandés.

J'apporterai mon aide à mes confrères ainsi qu'à leurs familles dans l'adversité.

Que les hommes et mes confrères m'accordent leur estime si je suis fidèle à mes promesses ; que je sois déshonorée et méprisée si j'y manque.



## Résumé

Du fait de l'amélioration du diagnostic et de la prise en charge thérapeutique, l'espérance de vie des patients avec lupus érythémateux systémique (LES) a augmenté au cours des dernières décennies. Cependant, cette population est toujours caractérisée par une morbi-mortalité élevée. Certaines manifestations cliniques, plus fréquemment observées à un âge avancé en population générale telles que événements cardio-vasculaires, cancers ou infections, apparaissent fréquemment à un âge plus précoce, soulevant l'hypothèse d'une accélération du processus de vieillissement chez les patients avec LES. Dans la première partie de ce travail, nous avons analysé les données de la littérature concernant les sept piliers du vieillissement (*i.e.* dommages macromoléculaires, métabolisme, cellules souches, proteostasis, adaptation au stress, inflammation et épigénétique) chez les patients avec LES, et exploré les interconnexions possibles entre ces deux cadres. Dans la seconde partie de ce travail, nous avons appliqué des biomarqueurs de méthylation de l'ADN, qui sont les biomarqueurs du vieillissement les plus robustes actuellement, à des données de patients avec LES. Nous avons inclus dans notre analyse deux bases de données distinctes comprenant 30 et 55 individus respectivement. Pour chaque patient LES et chaque contrôle, nous avons calculé son âge épigénétique et une mesure d'accélération de l'âge biologique, selon le modèle d'horloge épigénétique d'Horvath. Nous n'avons pas observé d'argument en faveur d'une accélération du vieillissement biologique selon cette mesure chez les patients avec LES, puisqu'il n'y avait pas de différence statistiquement significative entre les mesures d'accélération de l'âge épigénétique des patients et des contrôles.

## Abstract

Thanks to improvements in diagnosis, therapeutics and medical care, lifespan of systemic lupus erythematosus (SLE) patients has remarkably increased over the last decades. However, mortality ratio are still high in this population. Some clinical manifestations, more commonly found during old age in the general population such as cardiovascular events, cancers or infections, appear frequently at a younger age, raising the question of the presence of an acceleration of the aging process in SLE patients. In the first part of this work, we reviewed and compared literature evidence on the seven pillars of aging (*i.e.* macromolecular damage, metabolism, stem cells, proteostasis, adaptation to stress, inflammation and epigenetics) evaluated in SLE patients, and explored possible interconnections between these two frameworks. In the second part of this work, we applied DNA methylation biomarkers, which are the most robust biomarkers of age developed so far, in publicly available datasets of SLE patients. We included in our analysis two distinct DNA methylation datasets of 30 and 55 subjects respectively. For every SLE patient and control, we calculated their epigenetic age and a measure of epigenetic age acceleration, according to Horvath's epigenetic clock model. We did not observe any evidence of an accelerated biological aging according to this measure in SLE patients, as there was no statistically significant difference in the measure of epigenetic age acceleration between SLE patients and controls.