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Role of endoplasmic reticulum stress proteins in the pathogenesis of synovitis in osteoarthritis

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Thesis submitted to obtain the degree of Doctor in
Medical Sciences

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Academic year: 2025-2026

C'est avec beaucoup de joie que j'écris ces remerciements, car ils marquent l'aboutissement d'un projet commun, né de la collaboration entre le laboratoire et la clinique, deux mondes dont l'association reflète pleinement l'esprit translationnel de ce travail.

Ces cinq années au laboratoire m'ont fait découvrir un univers parallèle à la clinique, presque un cocon à côté de son tumulte. Elles m'ont profondément façonnée et m'ont permis de grandir, tant sur le plan humain que professionnel.

J'y ai rencontré des personnes formidables, engagées et toujours disponibles, qui savent rendre les journées plus légères même lorsque le travail est exigeant.

L'alternance entre six mois de clinique et six mois de laboratoire m'a appris à optimiser mon temps. Pour y parvenir, j'ai pu compter sur un encadrement bienveillant, aussi bien en clinique qu'en recherche, fait de patience, de confiance et d'absence de jugement.

Je sors de cette expérience grandie, avec encore plus d'ambition scientifique. Et si j'en suis là aujourd'hui, c'est surtout grâce à vous.

Cette thèse n'aurait pas été possible sans le soutien de ma promotrice et de ma co-promotrice, le Pr Ribbens et le Dr de Seny, qui m'ont accompagnée, tout au long de ce parcours.

Clio, merci de m'avoir permis de poursuivre mes manipulations au laboratoire en parallèle de ma formation clinique. Merci également pour votre disponibilité et votre réactivité dans l'organisation de la soutenance, malgré votre calendrier particulièrement chargé. Votre confiance et votre compréhension ont été essentielles pour mener ce projet à bien.

Domi, merci de m'avoir accordé une réelle autonomie dans la conception et la réalisation des expériences. Tu m'as laissé orienter et construire les manipulations tout en gardant un regard attentif et exigeant sur l'ensemble du travail. Cette liberté encadrée m'a énormément appris.

Merci à toutes les deux pour vos relectures précises du manuscrit et votre exigence constante quant à la rigueur scientifique et à la qualité rédactionnelle.

Je tiens également à remercier les membres de mon comité de thèse. Le Pr Louis, d'abord, vous qui m'avez soutenue au tout début de cette aventure en étant mon promoteur initial. Merci au Pr Marichal pour vos conseils en imagerie, toujours justes et pertinents, ainsi que pour votre capacité à saisir rapidement le concept du projet. Merci au Pr Jouret pour votre accueil chaleureux lors de mes six mois dans votre service de néphrologie, où je me suis immédiatement sentie à ma place, pour votre présence fiable et vos conseils avisés tout au long de ce projet de thèse, ainsi que pour l'impact déterminant de votre accompagnement dans mes démarches administratives pour mes projets futurs. Merci aussi au Pr Sellam pour nos échanges en post ACR qui m'ont réellement aiguillée pour l'écriture de ce manuscrit et merci au Pr Limaye d'avoir gentiment accepté de participer à l'évaluation de ce travail.

Je tiens également à remercier le Pr Michel Malaise de m'avoir guidée vers la recherche et le FNRS, de m'avoir soutenue dans mes candidatures avec enthousiasme, et de m'avoir convaincue d'y croire. Sans vous, je ne serais probablement pas là où je suis aujourd'hui.

Je remercie également le FNRS, l'Université de Liège, la Fondation Léon Frédéricq et CAP48 pour leur soutien financier. Un grand merci aussi aux différentes plateformes (Imagerie,

Immunohistologie, BHUL) pour leur aide indispensable, et en particulier à Gaëtan et Alex pour le temps consacré à mon projet et leurs conseils, notamment en bio-informatique et en imagerie, domaines dans lesquels je partais de loin.

Enfin, merci à Oli, Béa, Marie-Jo, Charline et Christian de m'avoir laissé la liberté de m'absenter de la clinique lorsque c'était nécessaire. Et surtout merci à Olichab et Suzon, mes deux co-assistants sûrs, d'avoir assuré les avis et le service sans jamais me le reprocher.

Ensuite, merci au laboratoire de gastro-rhumatologie. Parce que oui, les deux sont indissociables.

Pierre, ma première rencontre au laboratoire, dans la call room, pour ta "grosse" entorse. Ça a vite fini par se raconter des blagues quand on traînait au labo en fin de journée. Je pense sincèrement que tu es la personne qui me fait le plus rire dans la vie. Ton intelligence sociale et émotionnelle, ta capacité à cerner les gens et les situations, et à sortir LA blague au bon moment. Et Marty, l'homme le plus primé aux congrès, le cœur tendre. Vous avez toujours été de bons conseils pour m'apprendre les mécanismes de la recherche, m'aider à orienter la suite de mon projet, comprendre le pourquoi du comment, remettre en question mes résultats. Vous êtes mes têtes pensantes.

Charlotte et Juliette, les deux acolytes du fond, le call center du laboratoire, toujours les mots pour faire rire et la bonne humeur incarnée. Catherine, pour compléter cette dream team de gastro : merci pour ton efficacité, pour avoir toujours pris le temps de répondre à mes questions, le travail efficace par excellence.

Et puis la rhumato, parce que oui, c'est quand même la meilleure spécialité.

Sophie, merci pour ton écoute, ta capacité à toujours mettre les autres avant toi, ton énergie pour remotiver les troupes même dans les moments down. Merci de m'avoir aidée dans tout ce projet de A à Z et d'avoir été mon acolyte tout du long.

Zelda, merci pour ta maturité, ta positive attitude, ta bonne humeur permanente et le fait que tu ne refuses jamais d'aider le sanglier qui te prend au dépourvu.

Gaël, tu as été le premier à me faire découvrir la bench, à m'apprendre à manipuler et à utiliser les outils les plus bizarroïdes (QuPath, ImageJ). Bien que tu aies échoué à faire de moi la plus ordonnée sur la paillasse, je sais que dans le fond tu m'apprécies quand même. Merci aussi pour tous les débriefs sportifs possibles et inimaginables, que ça soit du tour de France ou de la NBA.

Christophe, toujours concentré derrière ton écran de 9h à 17h, j'ai eu la chance de découvrir une autre facette de toi au congrès de Brest. Merci pour ces discussions à refaire le monde autour de burgers et de couscous et pour m'avoir transmis les bases de l'informatique (en gros Excel) même si, on sait tous qu'officiellement, ce n'est pas ton job.

Céline, merci pour tes idées de génie pour pimper mon projet, pour avoir toujours eu la vision. Toujours un œil de loin, tu as su guider mon projet car tu le connais même mieux que moi.

Gene, merci pour ta bienveillance, ton calme, ta positivité, et tes gâteaux au chocolat, les meilleurs du monde. Tu t'es investie dans mon projet comme si c'était le tien. Merci surtout

pour ces derniers mois intensifs, à mes côtés pour les dernières manips, et pour tous ces mots doux qui ont illuminé mes journées.

Pauline, mon amie. Ça a commencé par discuter des techniques Picasso, ça a fini par parler de la vie en week-ends à la Table de Maxime, à Nieuport, dans un lit king size à Namur. Ce laboratoire m'aura fait rencontrer une vraie amie. Grâce à toi, j'ai pu, pour la première fois, être un peu la grande sœur, même si tu as rapidement pris ton envol. Ton franc-parler, ton ambition, ta détermination, ta mémoire incroyable, ta capacité à retenir tout ce que je te dis m'impressionnent (et ton montant OTP aussi).

Et enfin, ma famille. Mes parents, mon premier soutien, infaillible, la fiabilité à son apogée. Toujours le premier message pour demander comment ça s'est passé. Votre capacité à vous dévouer pour les filles et moi est sans doute ce qui me fascine le plus. Vous avez ce don de rendre simples des situations qui auraient pu être compliquées. Vous nous avez appris à nuancer les choses, à toujours chercher les solutions, à voir le positif, à nous faire notre propre opinion, se responsabiliser et à avancer avec confiance en nous. Et je mesure aujourd'hui la chance d'avoir grandi avec tout cela.

Mes grandes sœurs, Maud et Elsa, mes modèles et mes piliers. Tout ce que chacune pense, on le pense aussi. Même si ces années de voyage nous ont et vont nous séparer, il ne se passe pas un jour sans que l'on doive se donner de nouvelles de nos vies. Merci aussi de m'avoir donné deux frères, Loulou et Kandy, rencontrés chacun dans des circonstances particulières, la suite n'en a été que plus belle. Merci à vous quatre d'avoir toujours été mes premiers relecteurs dans tous mes plans professionnels. Et merci de m'avoir donné mes trois neveux et nièces chéris, Emy, Jude et Nina, que j'aime de tout mon cœur.

Merci aussi à mes ami(e)s, parce qu'à côté des études et du boulot, il y a toute l'aventure en parallèle: la guindaille, le baptême, les voyages, les runs, les marathons, les soirées à refaire le monde en conversations profondes. Merci à Cons, Margi, Claire, Marylou, Maud et Elise d'être toujours là, pour l'écoute attentive, pour ce groupe des 7 le plus chill du monde et c'est pour ça qu'on l'aime. Merci aussi à la team Rando, cette team qui s'est bâtie de façon aléatoire et qui est toujours au rendez-vous 1 fois par semaine.

Et merci à mes amies de secondaire, Cam, Marin et Auré pour votre fidélité incroyable et surtout à Tat, mon amie de toujours. Aux rires qui font mal aux côtes sur les bancs de maternelle, primaire et secondaire, et encore aujourd'hui dans les vignes du Beaujolais.

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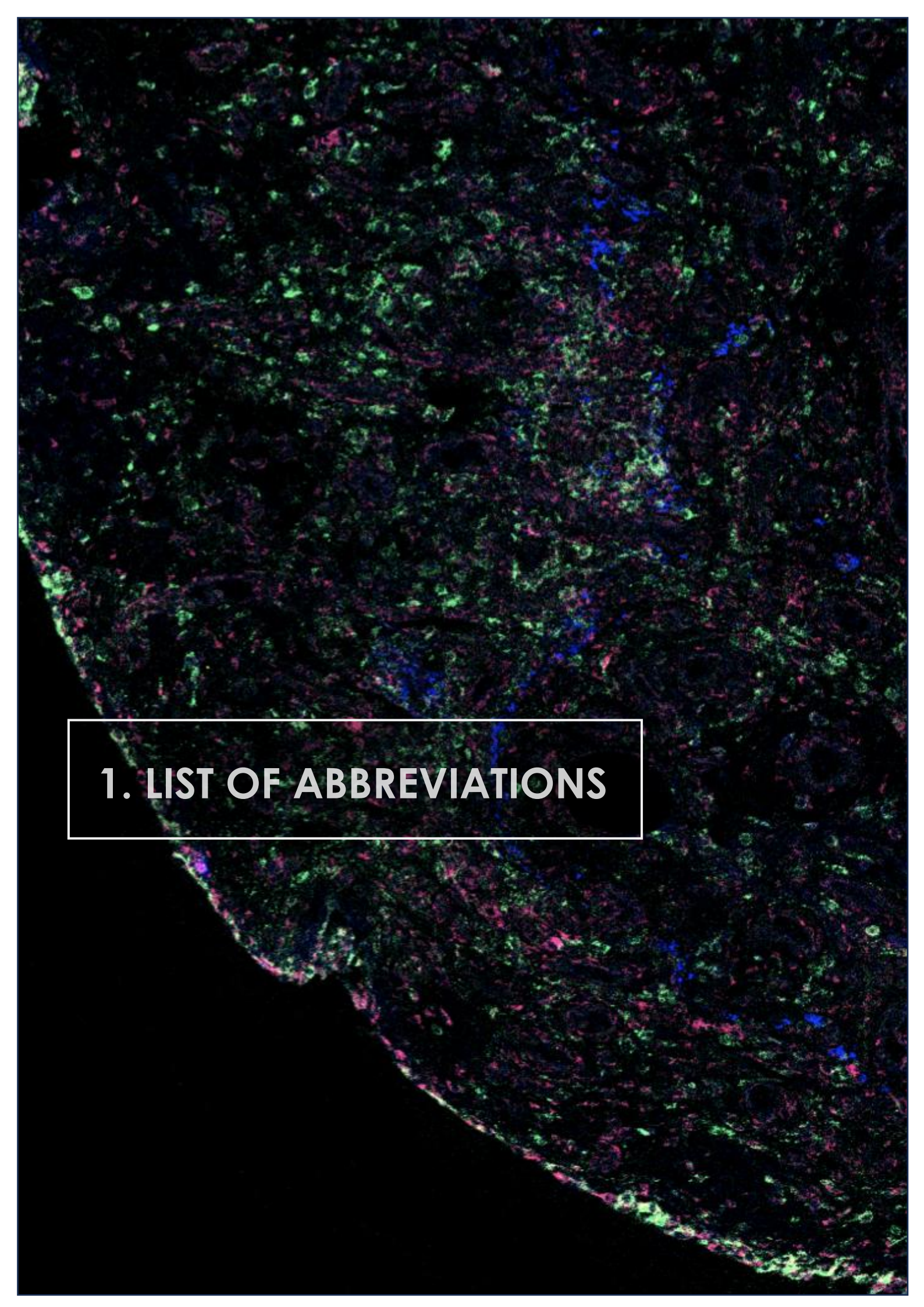
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AI-assisted language editing: ChatGPT (OpenAI) was used to improve the English wording of the manuscript. The scientific content, interpretation, and conclusions are the sole responsibility of the author.

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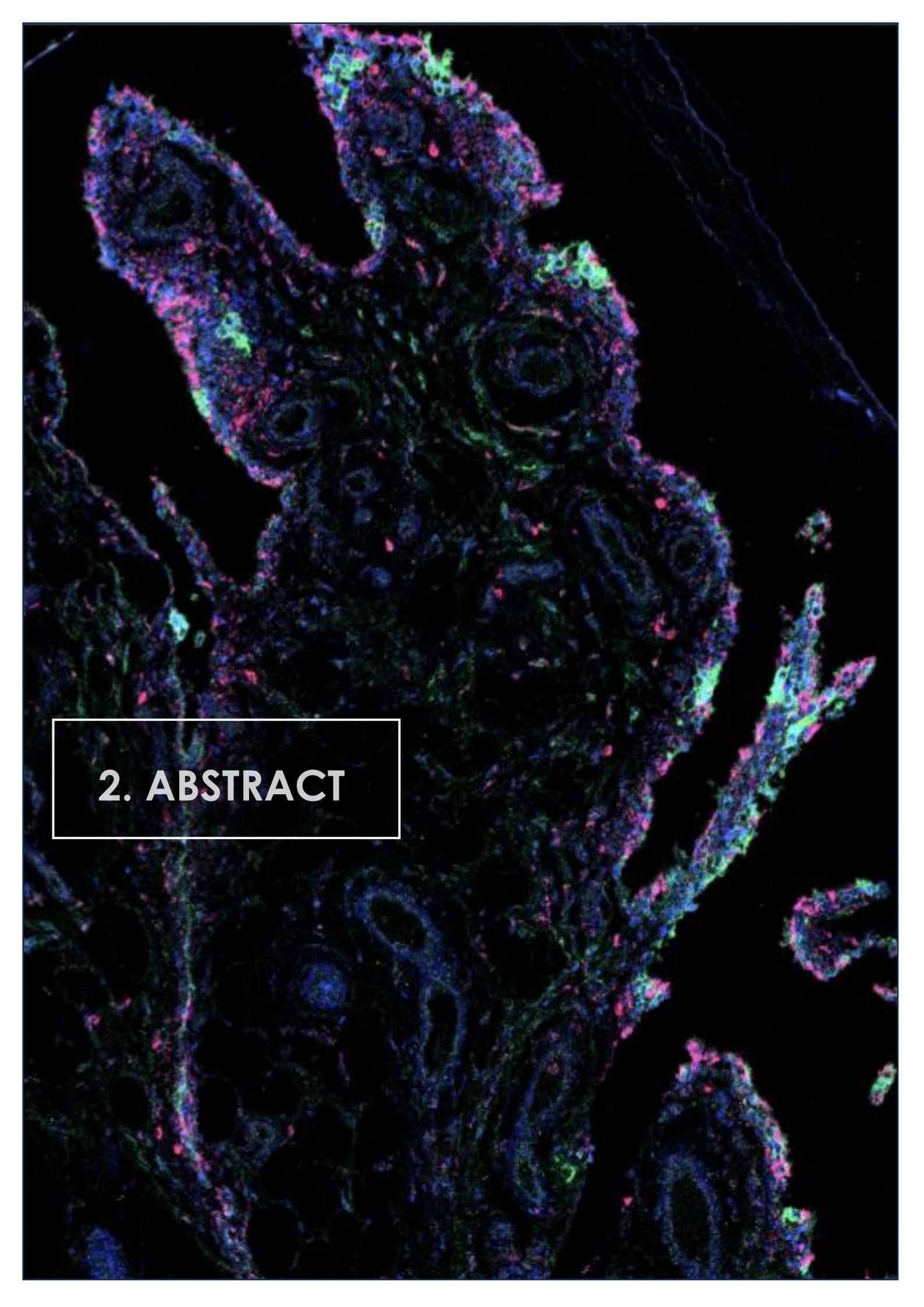
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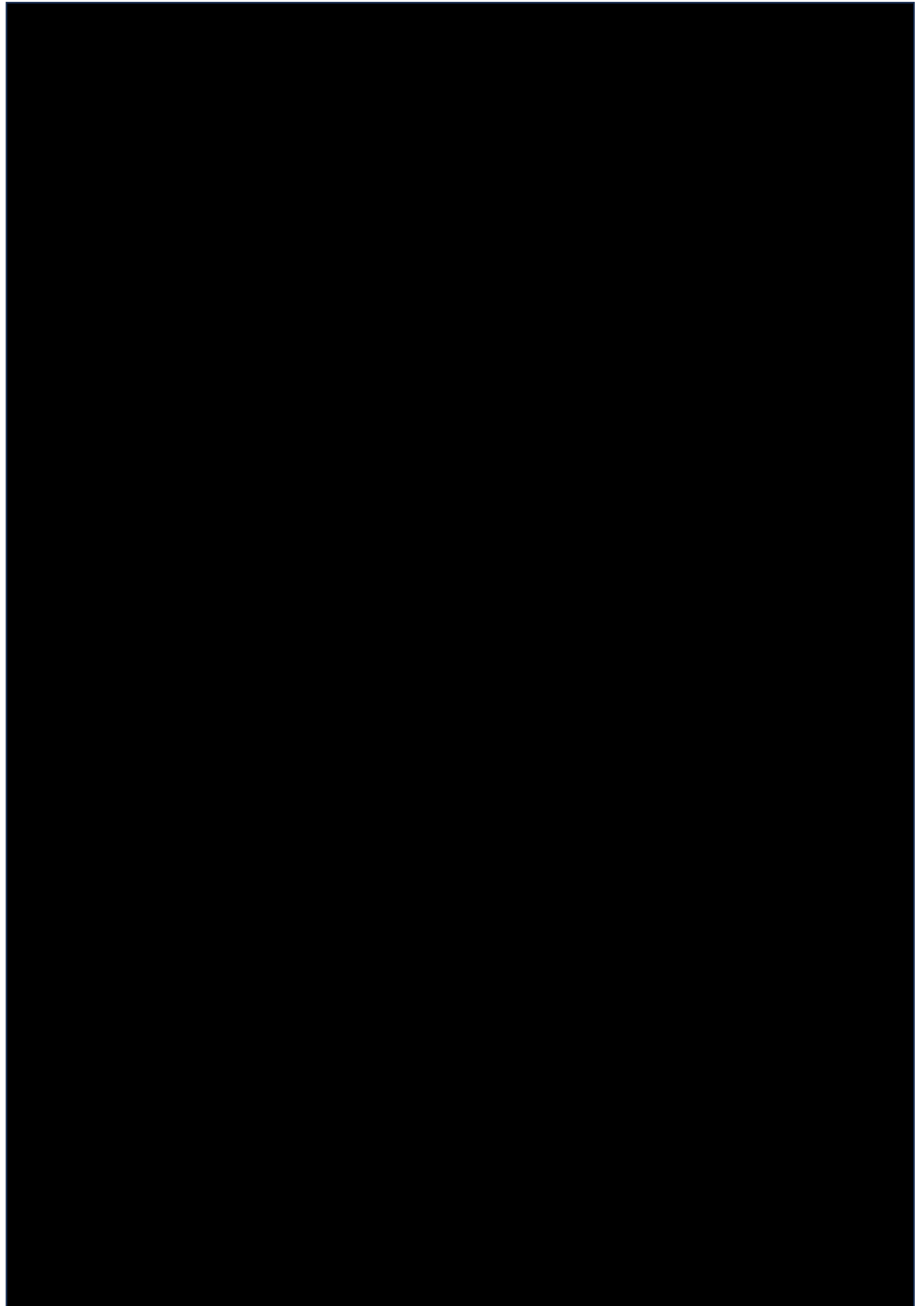
1. LIST OF ABBREVIATIONS

ACPA antibodies: anti-citrullinated protein antibodies
 ACR: American College of Rheumatology
 AGEs: advanced glycation end products
 ATF4: activating transcription factor 4
 ATF6: activating transcription factor 6
 BiP: binding immunoglobulin protein
 BMI: body mass index
 BML: bone marrow lesions
 CALR: calreticulin
 COX-2: cyclooxygenase 2
 CPPD: chronic pyrophosphate disease
 CRP: C-reactive protein
 DAMPs: damage-associated molecular patterns
 DMARD: disease-modifying anti-rheumatic drug; csDMARD: conventional synthetic DMARD, bDMARD: biologic DMARD, ts DMARD: targeted synthetic DMARD
 DMEM: dulbecco's modified eagle medium
 DMSO : dimethylsulfoxide
 DNAJB11: dnaJ heat shock protein family member B11
 eIF2 α : eucaryotic Initiation Factor 2 α
 EMT: epithelial-to-mesenchymal transition
 ER: endoplasmic reticulum
 ERP29: endoplasmic reticulum protein 29
 ESR: erythrocyte sedimentation rate
 EULAR: European Alliance of Associations for Rheumatology
 FBS: fetal bovine serum
 FGF: Fibroblast growth factor
 FLS: fibroblast-like synoviocytes
 FMT: fibroblast-to-myofibroblast transition
 GADD34: growth arrest and DNA damage-inducible protein
 GANAB: α -subunit of glucosidase II
 HA: hyaluronic acid
 HCQ: hydroxychloroquine
 HSP90B1: heat shock protein 90 beta family member 1
 HYOU1: hypoxia up-regulated 1
 IFP: infrapatellar fat pad
 IHC: immunohistochemistry
 I κ B: IkappaB
 IKK: I κ B kinase
 IL: interleukin
 IMC: imaging mass cytometry
 IN: inflammatory
 IRE1: inositol-requiring enzyme 1
 JNK: c-Jun N-terminal kinase
 JSN: joint space narrowing
 LMAN1: lectin mannose- binding 1
 MANF: mesencephalic astrocyte-derived neurotrophic factor
 MAPK: mitogen-activated protein kinase
 MLS: macrophage-like synoviocytes
 MMPs: metalloproteinases
 MRI: magnetic resonance imaging

NGF: nerve growth factor
NF- κ B: nuclear factor-kappa B
NIN: non-inflammatory
iNOS: inducible nitric oxide synthase
NSAIDs: nonsteroidal anti-inflammatory drugs
OA: osteoarthritis
OARSI: Osteoarthritis Research Society International
O/N: overnight
PBMCs: peripheral blood mononuclear cells
PDIA4: protein disulfide isomerase A4
PERK: protein kinase RNA-like ER kinase
PUFAs: polyunsaturated fatty acids
RA: rheumatoid arthritis
RF: rheumatoid factor
ROS: reactive oxygen species
RT: room temperature
siRNA: small interfering RNA
SMAD: Mothers against decapentaplegic homolog
TGF- β : transforming growth factor β
TXNDC5: thioredoxin domain containing 5
UPR: unfolded protein response
TNF- α : tumor necrosis factor- α
TRAF2: TNF receptor-associated factor 2
VEGF: vascular endothelial growth factor
XBP-1: X-box Binding Protein 1



2. ABSTRACT



Osteoarthritis (OA) is now recognized as a whole-joint disease in which synovial inflammation contributes to disease heterogeneity and progression. Endoplasmic reticulum (ER) stress has emerged as a key pathway in inflamed tissues. Nevertheless, the spatial distribution, cellular origin and functional contribution of ER stress proteins in OA synovitis remain poorly defined. This thesis aimed to characterize the expression of ER stress proteins across the inflammatory continuum of human synovitis, identify the synovial cell populations expressing these proteins, and explore whether selected ER stress proteins may participate in pathogenic mechanisms in OA fibroblast-like synoviocytes (FLS).

Based on previous proteomic data of the laboratory identifying eleven ER stress chaperones associated with synovial inflammation, their localization was investigated in human synovial biopsies from patients with OA, chronic pyrophosphate disease (CPPD), and rheumatoid arthritis (RA) using immunohistochemistry (IHC), imaging mass cytometry (IMC), and immunofluorescence (IF). These approaches were complemented by analyses in a collagenase-induced synovitis (CIS) mouse model. In parallel, primary OA FLS were used to study the modulation and secretion of chaperones under ER stress, inflammatory, and TGF- β stimulations, as well as the regulation of selected candidates, defined as the most strongly modulated proteins across ER stress, inflammatory, and fibrotic conditions, by unfolded protein response (UPR) pathways. Finally, functional assays based on siRNA-mediated silencing were performed to assess their potential contribution to fibrosis, inflammation, and proliferation.

Across human synovial tissues, ER stress proteins were predominantly confined to the lining layer in low-inflammatory samples and progressively extended into the sublining as inflammation increased. This pattern was confirmed in an independent cohort by IMC and was reproduced in the CIS model, in which several ER stress proteins were overexpressed in the synovial membrane compared with controls. In mice, distinct ER stress proteins correlated with inflammatory markers, synovial thickness, or the pro-fibrotic marker *Acta2*. Spatial analyses further indicated that ER stress proteins were mainly associated with CD55⁺ lining FLS in non-inflammatory (NIN) OA, whereas with increasing inflammatory severity they became preferentially co-expressed with CD34⁺CD31⁻ sublining FLS. In the extended OA cohort analyzed by IF, increasing inflammatory grades were also associated with an expansion of the CD34⁺PDIA4⁺ population within the sublining. A transitional CD55⁺CD34⁺CD31⁻ fibroblast subset was detected, and the inverse correlation observed between CD55⁺PDIA4⁺ lining FLS

and CD34+PDIA4+ sublining FLS suggests that PDIA4 may be involved in a shift between fibroblast phenotypes.

In vitro, OA FLS expressed both CD55 and CD34. ER stress induction upregulated several ER stress proteins and triggered secretion of a subset of them, including PDIA4. Inflammatory stimulation modulated BiP, HYOU1, MANF, and PDIA4, whereas TGF- β stimulation increased BiP, HYOU1, MANF, and DNAJB11. UPR inhibition experiments showed that HYOU1 and PDIA4 were regulated downstream of ATF6, while HYOU1 also appeared to be regulated by the IRE1 α pathway. Functionally, silencing of selected ER stress proteins did not significantly affect fibroblast-to-myofibroblast transition or IL-6 secretion under the tested conditions. In contrast, PDIA4 silencing significantly reduced TGF- β -induced FLS proliferation without affecting cell viability.

Overall, this work shows that the expression of ER stress proteins is spatially modulated during synovial inflammation and identifies FLS as a major cellular source in OA synovitis. Among these proteins, PDIA4 appears to be particularly associated with the expansion of CD34+ sublining fibroblasts and with synoviocyte proliferation, rather than with direct pro-fibrotic or pro-inflammatory responses in OA FLS under the conditions tested. These findings suggest a potential role for ER stress pathways, and especially PDIA4, in stromal remodeling during inflammatory OA synovitis.

French version

L'arthrose (OA) est désormais reconnue comme une maladie affectant l'ensemble de l'articulation, dans laquelle l'inflammation synoviale contribue à l'hétérogénéité et à la progression de la maladie. Le stress du réticulum endoplasmique (RE) s'impose par ailleurs comme un acteur clé de cette inflammation. Néanmoins, la distribution spatiale, l'origine cellulaire et le rôle fonctionnel des protéines associées au stress du RE dans la synovite arthrosique demeurent à ce jour insuffisamment caractérisés. Cette thèse vise à étudier l'expression des protéines du stress du RE, telles que les chaperonnes, en fonction du niveau inflammatoire de la synovite humaine, à identifier les populations cellulaires synoviales qui les expriment, et à explorer si certaines protéines du stress du RE peuvent participer à des

mécanismes pathogéniques dans les synoviocytes de type fibroblastique (FLS) issus de tissus arthrosiques.

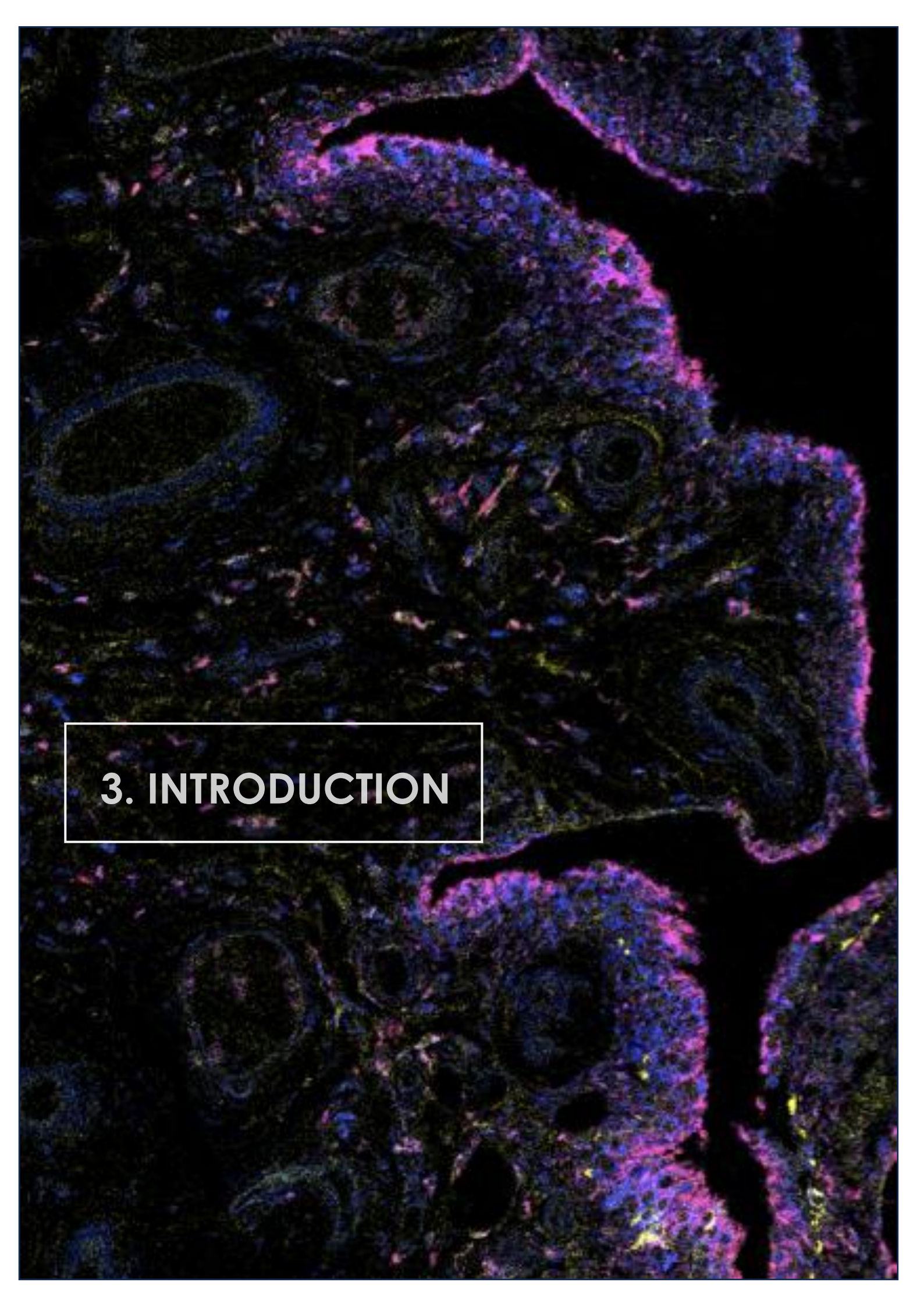
Sur la base d'une étude protéomique antérieure réalisée par spectrométrie de masse au laboratoire, onze protéines du stress du RE ont été identifiées comme associées à l'inflammation synoviale. Leur localisation a ensuite été étudiée dans des biopsies synoviales humaines provenant de patients atteints d'OA, de chondrocalcinose et de polyarthrite rhumatoïde (PR), en utilisant l'immunohistochimie (IHC), l'imagerie par cytométrie de masse (IMC) et l'immunofluorescence (IF). Ces approches ont été complétées par des analyses dans un modèle murin de synovite induite par la collagénase (CIS). En parallèle, des FLS primaires issus de membrane synoviale de patients atteints d'OA ont été utilisés pour étudier (i) la modulation de l'expression et de la sécrétion des chaperonnes dans des conditions de stress du RE, inflammatoires et sous simulation par TGF- β , ainsi que (ii) la régulation de certains candidats, définis comme les protéines les plus fortement modulées dans les conditions de stress du RE, inflammatoires et fibrosantes, par les voies de la réponse aux protéines mal repliées (UPR). Enfin, des essais fonctionnels basés sur la déplétion de leur expression par siRNA ont été réalisés afin d'évaluer leur contribution potentielle dans la fibrose, l'inflammation et la prolifération des OA FLS.

Dans les tissus synoviaux humains présentant un faible degré d'inflammation, nous avons principalement détecté les protéines du stress du RE au niveau de la couche bordante de la membrane synoviale, l'intima, qui est en contact direct avec la cavité intra-articulaire. En revanche, leur expression s'étend progressivement dans la subintima avec l'augmentation du degré d'inflammation. Ce profil a été confirmé dans une cohorte indépendante par IMC ainsi que dans le modèle CIS, pour lequel plusieurs protéines du stress du RE étaient surexprimées dans le tissu synovial comparativement aux témoins. Chez la souris, certaines protéines du stress du RE étaient corrélées à des marqueurs inflammatoires, à l'épaisseur synoviale ou au marqueur pro-fibrotique *Acta2*. Les analyses spatiales ont montré que les protéines du stress du RE étaient principalement associées aux FLS CD55⁺ de l'intima dans l'OA non inflammatoire, tandis qu'avec l'augmentation de la sévérité inflammatoire, elles étaient préférentiellement exprimées par les FLS CD34⁺CD31⁻ de la subintima. Dans la cohorte étendue d'OA analysée par IF, l'augmentation des grades inflammatoires était également associée à une expansion de la population CD34⁺PDIA4⁺ dans la subintima. La sous-population fibroblastique transitionnelle CD55⁺CD34⁺CD31⁻ ainsi que la corrélation inverse observée entre les FLS

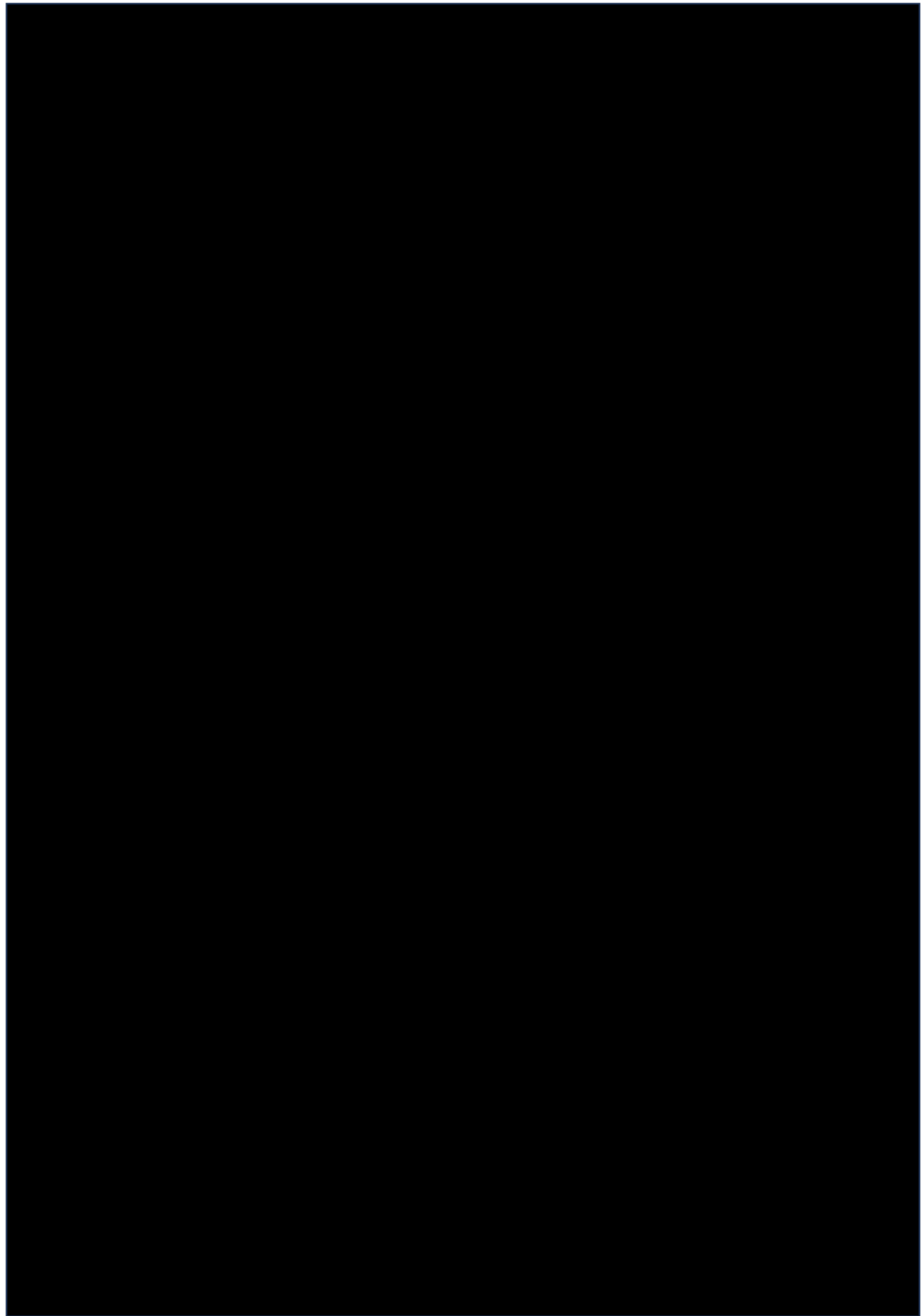
CD55⁺PDIA4⁺ de l'intima et les FLS CD34⁺PDIA4⁺ de la subintima suggèrent que PDIA4 pourrait être impliquée dans le changement phénotypique entre ces deux populations fibroblastiques.

In vitro, nous avons observé que les OA FLS exprimaient à la fois CD55 et CD34. L'induction du stress du RE a augmenté l'expression de plusieurs chaperonnes et a induit la sécrétion d'un sous-ensemble d'entre elles, incluant PDIA4. La stimulation inflammatoire a modulé BiP, HYOU1, MANF et PDIA4, tandis que la stimulation par TGF- β a augmenté BiP, HYOU1, MANF et DNAJB11. Les expériences d'inhibition des voies UPR ont montré que HYOU1 et PDIA4 étaient régulées en aval de la voie ATF6, tandis que l'expression de HYOU1 semblait également modulée par la voie IRE1 α . Sur le plan fonctionnel, la déplétion de certaines protéines du stress du RE n'a pas affecté significativement la transition fibroblaste–myofibroblaste ni la sécrétion d'IL-6 dans les conditions testées. En revanche, l'invalidation de PDIA4 a réduit significativement la prolifération des FLS induite par le TGF- β sans affecter la viabilité cellulaire.

Dans l'ensemble, ce travail montre que l'expression des protéines du stress du RE varie au cours de l'inflammation synoviale et que les FLS jouent un rôle majeur dans la synovite arthrosique. Parmi celles-ci, PDIA4 semble particulièrement associée à l'expansion des fibroblastes CD34⁺ de la subintima et à la prolifération des synoviocytes, plutôt qu'à des réponses directement pro-fibrotiques ou pro-inflammatoires des OA FLS dans les conditions étudiées. Ces résultats suggèrent un rôle potentiel des voies du stress du RE, et en particulier de PDIA4, dans le remodelage stromal au cours de la synovite inflammatoire de l'arthrose.



3. INTRODUCTION



3.1. Overview

Osteoarthritis (OA) is no longer regarded as a simple “wear-and-tear” degenerative disease of the cartilage, but rather as a whole-joint disease affecting all components of the joint, including cartilage, synovium, fat pad, subchondral bone, menisci and ligaments [1]. OA is a widespread joint disease among people, especially over the age of 65, and is a leading cause of joint pain, stiffness, and disability. Traditionally considered a degenerative disease driven by mechanical loading [2], obesity [3], or axial disorders [4], OA is now recognized as a multifactorial condition closely associated with components of the metabolic syndrome [5,6], including dyslipidemia [7] and insulin resistance. These metabolic alterations, together with inflammatory stimuli [cytokines or Damage-Associated Molecular Patterns (DAMPs)] [8], structural factors (age-related changes) [9], and other mechanical events (trauma, cartilage breakdown products, bone marrow lesions) [5], contribute to OA pathogenesis. Although OA has long been regarded as a non-inflammatory disease of the cartilage, the synovium also displays clear inflammatory features [1,8,10]. The synovial membrane is a connective tissue lining the joint cavity. It plays a crucial role in maintaining joint homeostasis and lubrication, and consists of two layers: the lining and the sublining. Several studies have demonstrated an association between endoplasmic reticulum (ER) stress and synovitis [11,12]. In our laboratory, we have previously identified by mass spectrometry 11 chaperone proteins of the ER stress (BiP, HYOU1, MANF, PDIA4, GANAB, HSP90B1, TXNDC5, DNAJB11, LMAN1, ERP29, and CALR), whose expression levels correlated with a histological inflammatory score established on synovial membranes obtained from patients with inflammatory joint diseases. These biopsies were obtained from patients with OA, chronic pyrophosphate disease (CPPD) or rheumatoid arthritis (RA), and exhibited a continuous degree of inflammation [13]. The ER is an intracellular organelle essential for proper protein folding and trafficking via chaperone proteins. Beyond their classic role in protein folding, several ER chaperones can also influence cell survival by enhancing inflammation and immunogenicity during pathological ER stress. Therefore, our study aims to investigate the impact of these 11 ER stress proteins on the initiation of fibrosis in arthritic diseases, with a particular focus on OA.

3.2. Osteoarthritis

3.2.1. Epidemiology and risk factors

OA affects approximately 7.6% of people worldwide, and its prevalence has increased by 30% over the past 30 years [14]. It is expected to rise by up to 100% in 2050 [15]. OA incidence is positively correlated with higher socioeconomic status, and significant disparities exist across geographic regions. Indeed, a higher prevalence is observed in the Asia-Pacific region and North America, while the lowest rates are found in Southeast Asia and sub-Saharan Africa [16]. Women not only represent the majority of affected individuals [17] but also tend to experience more severe symptoms [18]. The knee is the most commonly affected joint, with up to 45% of people likely to develop knee pain during their lifetime [19]. Other frequently affected joints include the hip, finger joints, particularly the thumbs, and the cervical and lumbar spine, predominantly in older populations [16]. Less commonly, OA can also affect the ankles, shoulders, elbows, wrists, and the temporomandibular joint [16].

According to the 2021 Global Burden of Disease (GBD) study, OA caused 21.3 million years lived with disability (YLDs) worldwide in 2021, compared to 8.92 million in 1990. This corresponds to a 139% increase, making OA the seventh leading cause of disability worldwide [20]. Consequently, absenteeism rates reached up to 14% [15]. A study conducted between 2011 and 2017 in Northern Europe showed that the incremental average annual direct costs of OA were up to €2,900 per patient, while indirect costs (productivity loss) could be as high as much as €13,800 per year [21].

Risk factors can be divided into those at the individual levels and those at the joint level (Figure 1) [16].

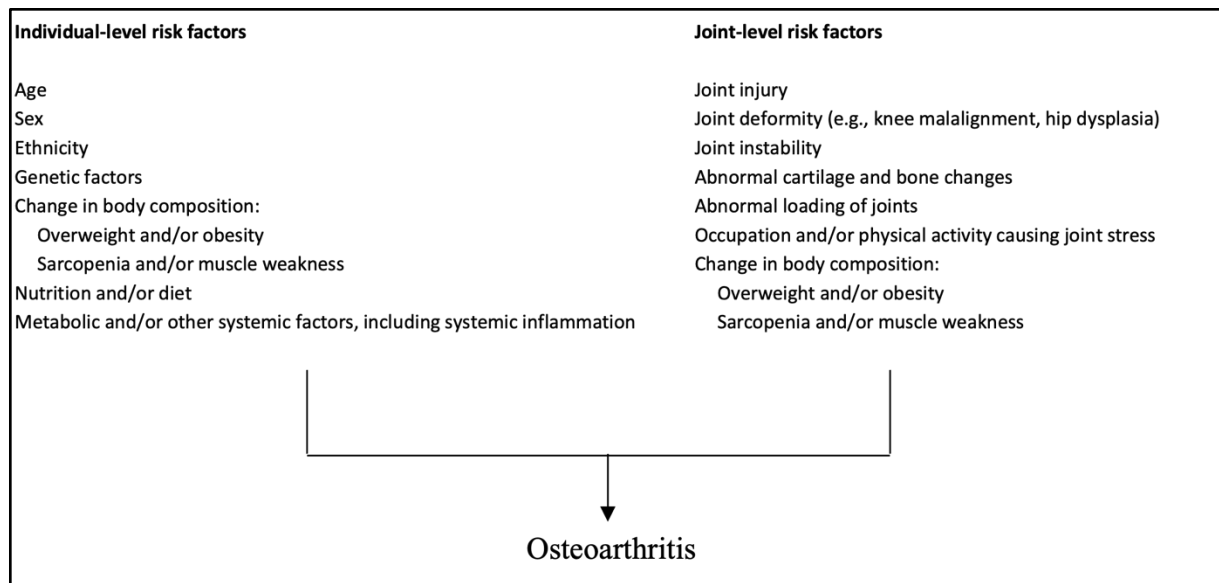


Figure 1. Risk factors of OA at the individual and joint levels [16].

- **Age:** with increasing age, there is not only a progressive accumulation of risk factors but also structural changes within the joint itself [16]. Aging is also associated with a higher prevalence of metabolic syndrome [22]. This process is partly driven by inflammaging, a low-grade chronic inflammatory state caused by the accumulation of inflammatory cytokines and reactive oxygen species (ROS), cellular senescence, mitochondrial dysfunction, impairment of autophagy, activation of the inflammasome, dysregulation of the ubiquitin–proteasome system, dysbiosis and a reduced regulatory capacity of T-cells [23]. Altogether, these mechanisms can contribute to fibroblast-like synoviocytes (FLS) activation and cartilage degradation [24].
- **Gender:** according to the Framingham Cohort, symptomatic hand OA affected 13% of men and 26% of women over the age of 70 [25]. Hormonal factors have been extensively investigated, with estrogens generally exerting anabolic and proliferative effects on chondrocytes, although their role in OA remains complex and partly contradictory [26]. Some studies suggest that postmenopausal women tend to develop more severe OA [27], whereas others have failed to confirm this association [28]. A recent review nonetheless supports the concept of a sex-specific susceptibility profile, in which risk factors such as BMI, atherosclerosis, and alcohol consumption disproportionately affect women [29,30]. Anatomical and biomechanical differences between sexes may also contribute to OA risk, including variations in joint anatomy and composition (cartilage thickness, joint surface area), alignment, muscle strength,

ligamentous laxity, and lifestyle-related mechanical loading [31,32]. Finally, differences in pain sensitivity and neuro-immune regulation appear to play a major role. Women have been reported to exhibit distinct neuro-immune signaling patterns linking joint and central nervous system inflammation, with coordinated monocyte chemoattractant protein 1 (MCP-1) levels across synovial fluid, serum, and cerebrospinal fluid that are not observed in men [32]. Moreover, pain severity correlates positively with IL-6 and IL-8 levels in synovial fluid but inversely with their concentrations in cerebrospinal fluid, potentially influencing symptom severity and disease risk [32]. Depression, which is more prevalent in women [33], may further amplify pain perception and symptom burden [34]. Sex-related differences also extend to pain management, as women tend to show greater clinical responses to non-pharmacological interventions such as superficial heat or cold therapy [35], and may experience enhanced analgesic effects of opioids in postoperative and chronic pain settings [36]. Despite these observations, the precise pathophysiological mechanisms underlying sex-related disparities in OA prevalence and severity remain incompletely understood.

- **Ethnicity:** Population-based data from the United States indicate marked racial and ethnic disparities in overall arthritis burden. Although the prevalence of arthritis is higher among non-Hispanic White and American Indian/Alaska Native adults, non-Hispanic Black and Hispanic adults living with arthritis are more frequently affected by severe joint pain [37]. Focusing more specifically on OA, African Americans appear to experience a higher prevalence and greater radiographic severity of knee OA than Whites, and OA-related pain and functional impairment also tend to be more pronounced in this population [38]. Several studies have reported greater pain severity among African American patients with knee OA compared with Caucasian Americans [39,40]; however, these findings are not entirely consistent, as one recent study showed no significant differences in self-reported pain severity between African American and Caucasian men with OA after stratification by radiographic disease severity [41].
- **Genetic factors:** heritability is estimated at around 40% for knee OA and up to 60% for hand and hip OA [42]. This genetic contribution reflects a polygenic architecture driven by multiple germline variants rather than a Mendelian inheritance pattern. Genome-wide association studies have identified susceptibility loci in genes involved in joint

development, cartilage differentiation, and endochondral ossification. Distinct genes contribute through different types of variants. Growth and Differentiation Factor 5 (GDF5), a key regulator of joint morphogenesis, is mainly associated with single-nucleotide polymorphisms, including missense variants [43]. Mothers Against Decapentaplegic homolog 3 (SMAD3), a central mediator of Transforming Growth Factor β (TGF- β) signaling and chondrocyte homeostasis, is primarily linked to non-coding regulatory variants. In contrast, Parathyroid Hormone-Like Hormone (PTHrP) and Runt-related transcription factor 2 (RUNX2), which regulate chondrocyte proliferation and maturation, harbor coding variants such as missense and frameshift mutations that may alter cartilage development and joint biomechanics [24]. Additional loci involved in extracellular matrix organization, including Carbohydrate Sulfotransferase 11 (CHST11) and Collagen beta(1-O)galactosyltransferase 2 (COLGALT2), further support a genetic contribution to OA susceptibility [44]. While epigenetic mechanisms, such as altered DNA methylation of COLGALT2, may modulate gene expression and disease risk, they should be considered modifiers rather than determinants of heritability.

- Obesity/sarcopenia: there is a clear relationship between obesity, as measured by BMI, and the incidence of OA. This risk is even higher in women compared to men, supporting the idea of a gender-based difference in susceptibility to obesity-related OA [45,46]. Obesity increases compressive forces on the joint. This is partly because the forces of the quadriceps, hamstrings, and gastrocnemius muscles must increase to stabilize and propel the joint during movement. This leads to further cartilage and meniscal degradation [47–49]. The association between obesity and OA is also observed in non-weight-bearing joints, which is explained by the role of adipose tissue in releasing adipokines or inflammatory cytokines, such as leptin [50]. Consistently, central obesity assessed by waist circumference has been shown to increase the risk of OA independently of BMI, further supporting a metabolic and inflammatory contribution of visceral adiposity beyond mechanical joint loading [46]. In addition, a subgroup of interest is sarcopenic obesity in which loss of muscle mass may lead to imbalance and altered joint loading, particularly in weight-bearing joints such as the knees [51]. This muscle loss could be related to the increase of inflammatory cytokines levels driven by leptin secretion [52]. A study demonstrated in 2022 that patients with

sarcopenic obesity have a higher risk of developing OA than those with non-sarcopenic obesity, adding yet another dimension to this relationship [53].

- Metabolic syndrome: hypertension, dyslipidemia, hyperglycemia, and insulin resistance, even after adjustment for BMI, are all contributors to OA through the promotion of pro-inflammatory cytokine secretion, oxidative stress, low-grade chronic inflammation, or the accumulation of Advanced Glycation End products (AGEs) in synovium, cartilage or subchondral bone [54–56]. The role of obesity in this process has been described above.
- Diet: epidemiological data from the Osteoarthritis Initiative (OAI) cohort suggest that dietary patterns may influence both the incidence and progression of knee OA. Adherence to a Western dietary pattern, characterized by high consumption of processed meats, refined grains, red meat, fried foods, and sugar-containing beverages, was associated with increased radiographic and symptomatic progression of knee OA, whereas a prudent dietary pattern, rich in fruits, vegetables, legumes, fish, and whole grains, was associated with reduced disease progression [57]. Similarly, individuals in the highest quartile of adherence to a Western diet showed an increased risk of incident knee OA, while a prudent dietary pattern was associated with a lower risk. However, these associations were attenuated after adjustment for baseline BMI [58]. At the nutrient level, omega-6 polyunsaturated fatty acids (n-6 PUFAs) have been associated with synovitis and bone marrow lesions (BML) [59,60], whereas omega-3 PUFAs, through their anti-inflammatory properties, may exert protective effects by reducing cartilage degradation, particularly in the patellofemoral compartment [60].
- Cholesterol: both cross-sectional and longitudinal studies have shown links between cholesterol increase and OA, likely through secondary cytotoxic effects [61]. Cholesterol can indeed accumulate within cartilage, and its excess may contribute to cartilage degradation [62].
- Joint injury/deformity/instability: a direct injury to joint tissues can cause OA, while indirect factors like poor alignment or abnormal load distribution may also promote disease [63]. For example, severe knee laxity, although it might itself be a consequence of OA, as well as varus or valgus malalignment, muscle atrophy post injury, leg length

inequality can contribute to disease development [63–65]. It is important to note that joint injury does not solely affect the cartilage directly but also damages adjacent tissues such as the synovium, meniscus, and infrapatellar fat pad (IFP), which can subsequently release inflammatory mediators that further harm the cartilage [66].

- Abnormal cartilage and bone changes: large or small cartilage defect, osteophytes, osteoporosis, bone marrow lesions [16].
- Occupational: mechanical exposures have been investigated as potential risk factors for OA, with varying levels of evidence depending on the type of exposure and joint involved. Moderate to strong evidence supports an association between cumulative physical loads and knee OA, particularly when combining activities such as kneeling, squatting, and bending across men and women [67,68]. For hip OA, lifting-related activities have been associated with increased risk, and moderate evidence also exists for cumulative mechanical loading and exposure to whole-body vibration [67,69]. Occupational vibration exposure is one of the most consistently supported risk factors for OA. Prolonged exposure to mechanical vibration, such as full-body vibration in vehicle operators or repetitive tool-related vibration in dentists, has been associated with an increased risk of joint degeneration, including hand OA among dental professionals [67,70]. In contrast, the evidence regarding other physically demanding occupational tasks, such as isolated kneeling, squatting, climbing, or prolonged standing, remains more heterogeneous. Although several meta-analyses suggest increased odds of knee OA compared with sedentary work, inconsistencies in exposure definitions, variability in OA outcome measures, and incomplete adjustment for confounders limit definitive causal conclusions [68,69].

3.2.2. Classification

OA is traditionally classified as primary (idiopathic) or secondary (with an underlying cause) (Table 1) [71,72]. Primary OA most commonly involves weight-bearing and mechanically stressed joints such as the knees, hips, lumbar spine, and the first carpometacarpal joint. By contrast, secondary OA tends to affect more atypical sites, including the ankles, shoulders, elbows, wrists, and the temporomandibular joint, often in the setting of trauma or other predisposing factors [16].

Primary
Secondary
Post traumatic (fracture, infection, joint surgery)
Congenital disorders (congenital hip dislocation, chondral dysplasia)
Metabolic (calcium crystal deposition, haemochromatosis, diabetes, acromegaly, Paget's disease)
Inflammatory (inflammatory arthritis, e.g. rheumatoid arthritis; septic arthritis)
Other bone and joint disorders (avascular necrosis, neuropathic Charcot joints)

Table 1. Traditional classification of OA by cause; adapted from [71].

Earlier OA classifications were primarily phenotype-based and focused on the affected joint tissues, whereas more recent approaches emphasize endotypes that reflect the underlying biological mechanisms of OA; however, these endotypes are not mutually exclusive and rather overlap, highlighting the complex and heterogeneous nature of OA (Figure 2) [73].

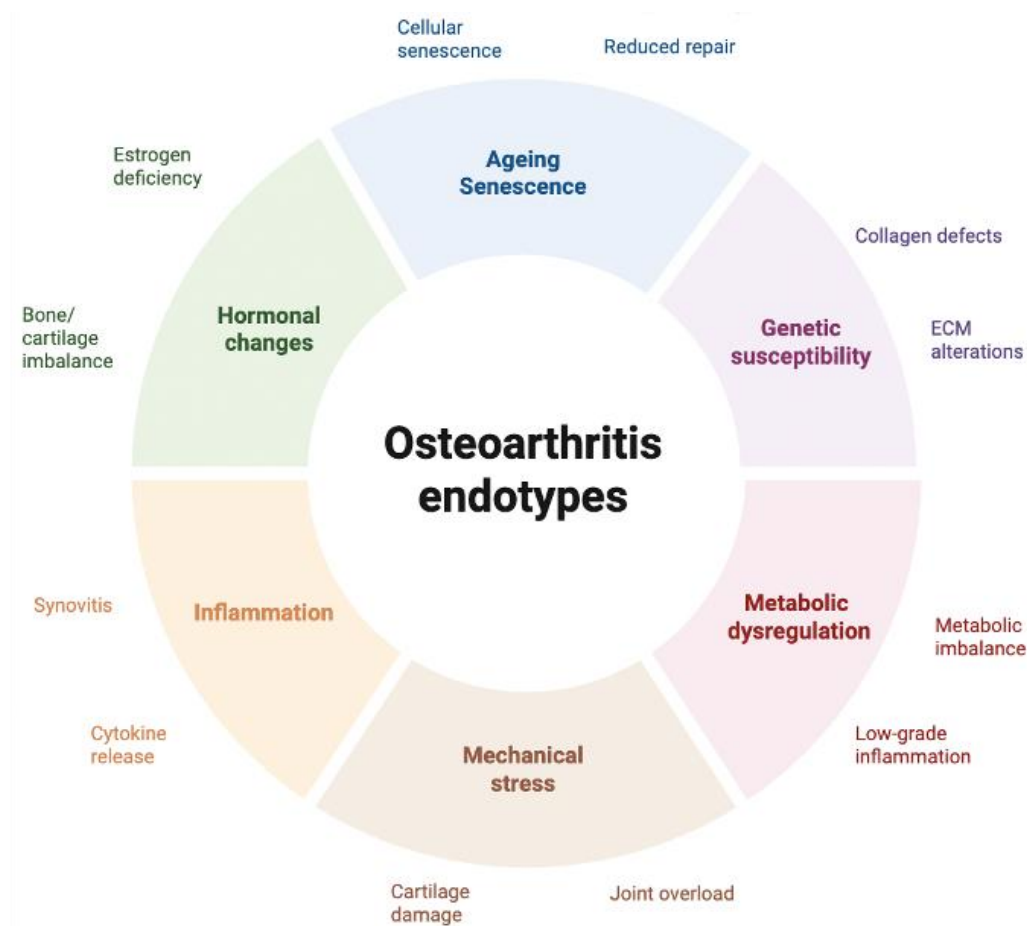


Figure 2. Classification of OA according to its endotype; adapted from [73], made with BioRender (figure creation software). OA: osteoarthritis; ECM: extracellular matrix.

More recently, advances in systems biology, high-dimensional analytics, and omics technologies have expanded the concept of OA endotypes toward the identification of distinct molecular signatures. Clustering approaches based on microRNA profiles from plasma, synovial fluid, and urine, as well as plasma metabolites, allow OA patients to be stratified into molecular clusters, with the aim of determining differences in treatment response and assessing whether specific molecular signatures can predict disease progression and the risk of total knee arthroplasty [74].

3.2.3. Clinical features

OA diagnosis is based on clinical symptoms, mostly joint pain and stiffness [75]. According to the EULAR criteria, knee OA is defined as occurring in patients over 40 years old who experience joint pain related to movement, morning stiffness lasting less than 30 minutes, and functional limitations. A diagnosis is confirmed if, in addition, at least one of the following findings is present on examination: crepitus, restricted range of motion, or bony enlargement. When all these criteria are met, the probability of having radiographic knee OA reaches 99% [76]. Similarly, the criteria established by the ACR define knee OA as the presence of knee pain associated with at least three of the following features: age over 50 years, morning stiffness lasting less than 30 minutes, crepitus, bony tenderness, bony enlargement, and absence of palpable warmth [77]. These classification criteria are widely used in research settings to standardize patient populations.

For hand OA, the more commonly used ACR classification criteria define the disease by the presence of hand pain, aching or stiffness, together with at least 3 of the following: hard tissue enlargement of ≥ 2 of 10 selected joints, hard tissue enlargement of ≥ 2 distal interphalangeal joints, fewer than 3 swollen metacarpophalangeal joints, and deformity of at least 1 selected joint [78]. More recently, the EULAR group updated its diagnostic framework [79]. Although not used in daily practice, these criteria aim to define more homogeneous patient populations for research and clinical trials. Clinicians are first advised to identify pain, aching, or stiffness affecting at least one relevant joint on most days over the past six weeks, not explained by acute injury or other conditions such as crystal arthropathies or systemic inflammatory diseases. If fulfilled, a scoring system is applied based on age (0–3), morning stiffness duration (0–2), number of joints with osteophytes (0–4), joint space narrowing (0–3), and symptom–structure concordance (+3 if present) [79].

Unlike inflammatory arthritis, OA pain is typically worsened by joint use and relieved by rest. This type of pain is called mechanical pain, and it is often the most troublesome symptom. In contrast, stiffness usually improves with movement. However, night pain can occur as well, interfering with sleep and leading to fatigue, reduced well-being, and increased pain sensitivity. Temporal and seasonal variations in OA pain have also been reported, like other arthropathies [75].

Flares can occur, presenting with painful joint swelling that may mimic inflammatory, infectious, or microcrystalline arthritis, but usually with low synovial white blood cell count, normal glucose, and absence of crystals [72,80]. Additionally, painful periarticular soft tissue lesions, such as pes anserine bursitis or greater trochanteric pain syndrome, may coexist with large-joint OA [81].

The severity of joint pain is linked to the extent of lesions observed on MRI, particularly subchondral bone damage, synovitis, BML and meniscal tears [82–84].

3.2.4. Anatomy

OA is a progressive destructive disease that affects the entire joint and primarily involves cartilage degradation. Although other tissues can also be affected, including the synovial membrane (synovitis), subchondral bone (bone marrow lesions), the infrapatellar fat pad (IFP - inflamed and fibrotic), ligaments, muscles, and frayed menisci, it is the damage to the cartilage and subchondral bone that mainly drives radiographic changes, including joint space narrowing (JSN), subchondral bone sclerosis, subchondral cysts and osteophyte formation (Figure 3) [16].

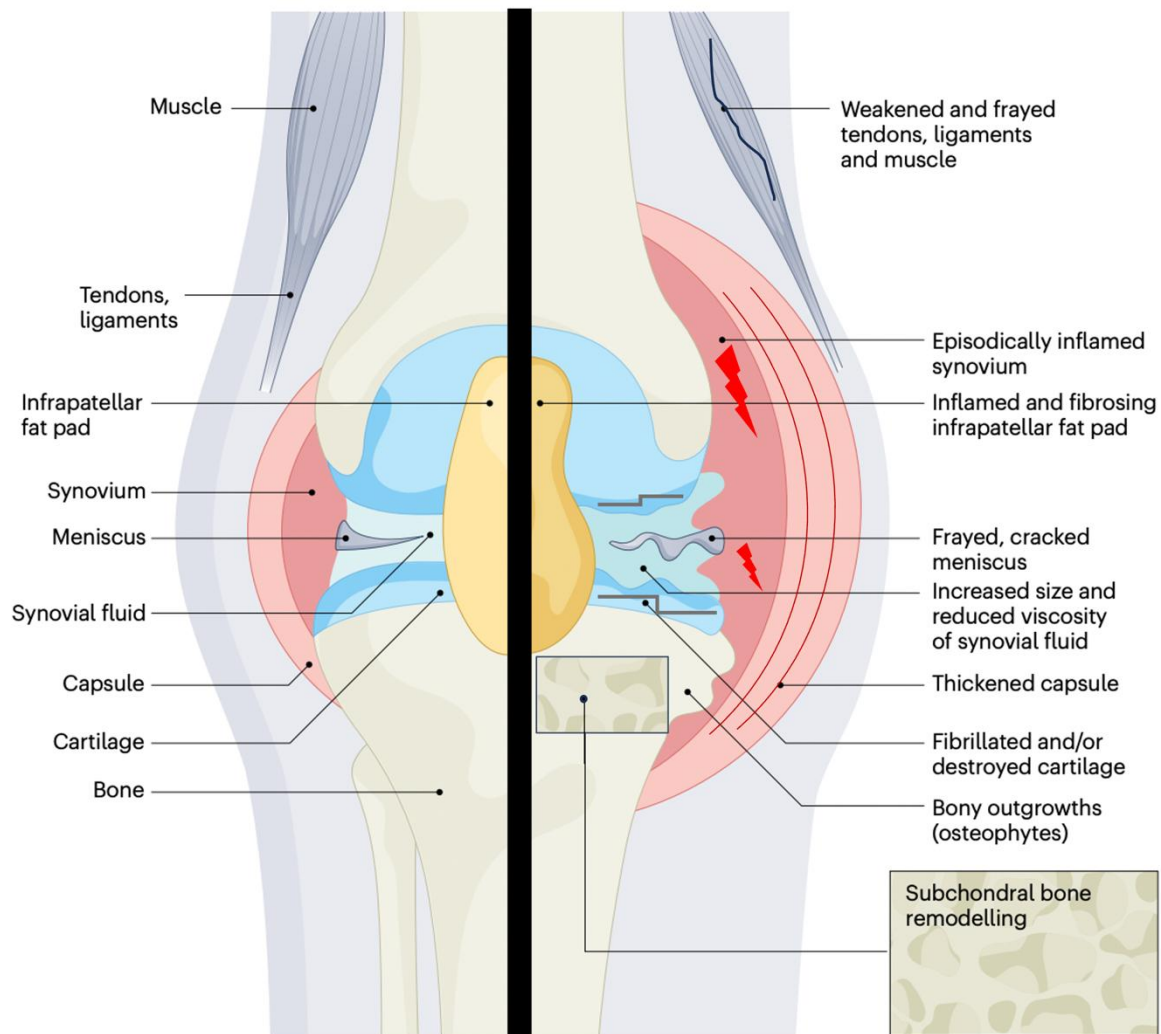


Figure 3. A comparison of a healthy joint with an OA joint. In OA, nearly all joint tissues exhibit pathological changes (cartilage degradation, synovial and IFP inflammation and fibrosis, subchondral bone remodeling, meniscal and ligament damage); adapted from [16]. OA: osteoarthritis; IFP: infrapatellar fat pad

3.2.5. Cartilage degradation

Cartilage degradation is a central hallmark of OA pathophysiology and represents a key driver of disease initiation and progression. The articular cartilage is a hyaline connective tissue that covers the ends of bones in diarthrodial joints and lies directly above the subchondral bone, together forming the osteochondral unit. Its primary functions are to provide a smooth, low-friction surface for joint movement and to absorb mechanical shock. Cartilage is an aneural and avascular tissue composed of approximately 65–80% water, 20–40% extracellular matrix, and only 1–5% chondrocytes. Owing to its avascular nature, cartilage relies primarily on nutrients supplied by the synovial fluid produced by the synovial membrane, with additional nutritional

support provided by diffusion from the subchondral bone. Beyond its nutritional role, the subchondral bone also contributes to the mechanical stability of the joint, indicating that alterations in the subchondral bone microenvironment can directly influence cartilage metabolism and homeostasis [85,86].

During the initiation stage of OA, quiescent articular chondrocytes exposed to repetitive microtraumatic mechanical insults become activated and re-enter a proliferative state in an attempt to compensate for early extracellular matrix loss. This adaptive response is accompanied by the onset of hypertrophic differentiation, marked by the expression of collagen type X (COL10A1) and the transcription factor RUNX2 [87,88]. In parallel to hypertrophic differentiation, a subset of chondrocytes can undergo phenotypic transition toward fibroblast-like or chondro-myofibroblast-like cells, contributing to the emergence of a fibrocartilage-like phenotype [89]. At this early stage, chondrocytes also acquire an inflammatory phenotype and begin to secrete pro-inflammatory cytokines, notably Tumor Necrosis Factor- α (TNF- α) and Interleukin-1 β , which initiate a shift toward catabolic signaling and promote the induction of matrix-degrading enzymes such as MMP-13 and aggrecanases (including ADAMTS-4 and ADAMTS-5) [90]. As a result of this proteolytic imbalance, OA cartilage shows a progressive downregulation of key articular cartilage markers, including Lubricin (PRG4), Aggrecan (ACAN), and Collagen type II (COL2A1) [87]. Interestingly, while alterations in TGF- β signaling in OA have been associated with reduced ALK5 activity and decreased Smad2/3 phosphorylation, which normally restrain chondrocyte hypertrophy [91], Deroyer *et al.* reported that TGF- β stimulation in chondrocytes robustly activates the canonical p-Smad2/3 pathway and promotes the expression of pro-fibrotic mediators [89]. Consistent with the context-dependent effects of TGF- β , short-term signaling promotes an anabolic response, whereas sustained exposure favors hypertrophic differentiation and catabolic activity [92].

As OA progresses, focal proteoglycan depletion exposes the underlying COL2A1 network, increasing its susceptibility to proteolytic cleavage and altering the biomechanical cues perceived by chondrocytes. This change activates the Discoidin Domain Receptor 2 (DDR2), further amplifying hypertrophy-associated signaling and sustaining matrix-degrading enzyme expression, thereby accelerating cartilage breakdown [88]. In advanced disease, cartilage is characterized by clusters of chondrocytes that remain in a highly catabolic state, while other chondrocytes undergo apoptosis, leading to progressive cellular loss. These cellular clusters may also include proliferating chondrocytes exhibiting myofibroblast-like features, further

contributing to matrix remodeling and fibrocartilaginous tissue formation [89]. Together, persistent matrix degradation and reduced cellularity markedly impair the reparative capacity of cartilage and drive irreversible structural joint damage [88] [99] (Figure 4).

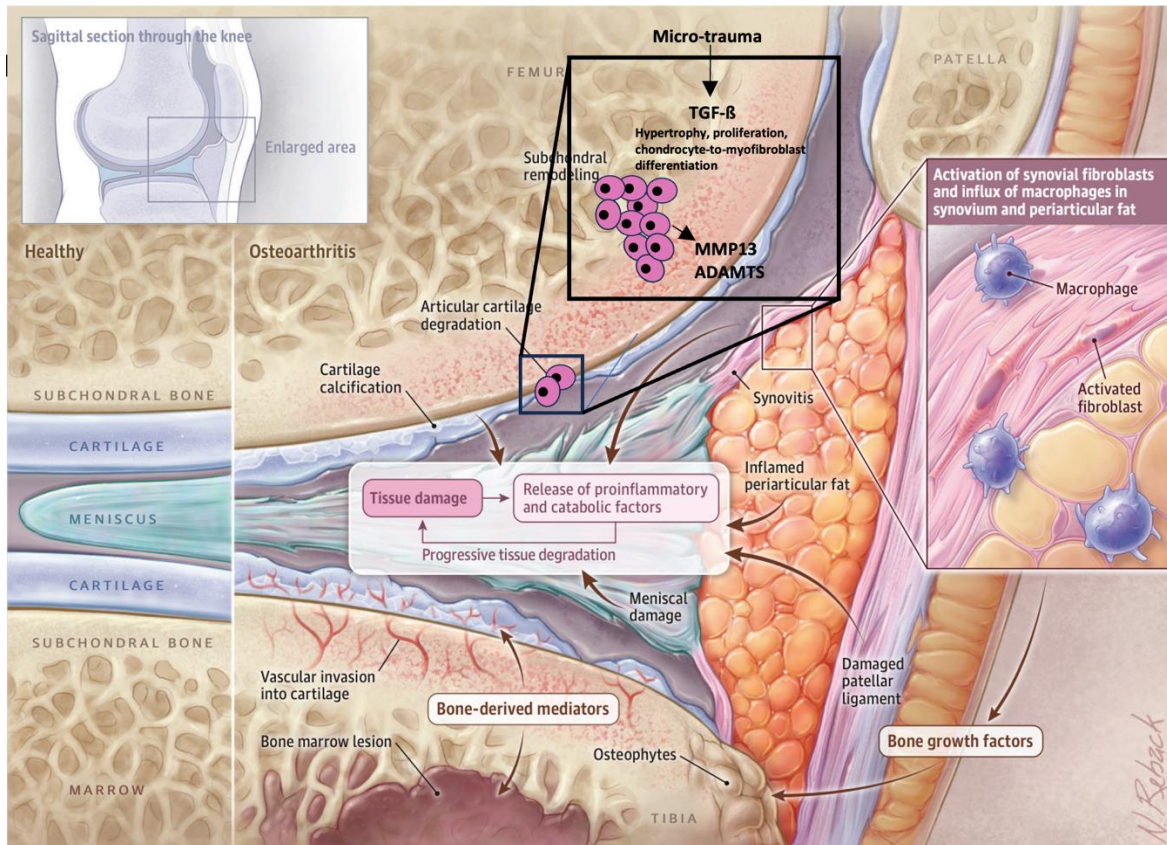


Figure 4. Multifactorial and dynamic processes driving OA progression. Cartilage degeneration in OA is not a linear process but results from complex interactions between chondrocytes, synovial inflammation, and subchondral bone remodeling. Early mechanical stress and micro-trauma induce chondrocyte hypertrophy, proliferation and a catabolic shift, associated with increased production of MMP-13 and ADAMTS, as well as pro-inflammatory cytokines (e.g., $\text{TNF-}\alpha$, $\text{IL-1}\beta$). [88]. OA: osteoarthritis. Adapted from [93].

Beyond cartilage-intrinsic mechanisms, OA must also be considered as a disease of the osteochondral unit, in which close functional coupling exists between cartilage and the underlying subchondral bone. One of the earliest pathological features affecting this unit is the development of BML. It consists of microfractures in the subchondral bone induced by abnormal and persistent mechanical loading, which may even precede cartilage damage [85,94,95]. In parallel, neovascularisation develops within the subchondral tissue, accompanied by increased bone volume and reduced mineral density, a process referred to as subchondral bone sclerosis [85]. This vascular and structural remodeling promotes osteophyte formation,

which is thought to represent an adaptive response aimed at redistributing joint load by increasing the articular surface area.

Subchondral bone remodeling is driven by interactions between osteoblasts, osteoclasts and bone marrow-derived mesenchymal stem cells. In damaged subchondral bone, the latter cells, under TGF- β stimulation, migrate from the bone marrow and differentiate into immature osteoblasts. They adopt a pro-inflammatory and pro-catabolic phenotype, expressing inflammatory cytokines, MMP-1, TGF- β and prostaglandin E2, which stimulate chondrocytes to increase MMP-13 expression and decrease aggrecan synthesis, further exacerbating cartilage degradation [85]. In addition, immature osteoblasts promote osteoclast activation through the receptor activator of nuclear factor kappa-B (ligand) RANK/RANKL system, leading to resorption of the subchondral plate and enabling osteoclast invasion into the non-calcified cartilage, further contributing to cartilage degradation. This pathway can also be amplified by B cells, which represent an additional source of RANKL [96]. Beyond this, B cells can impair bone fracture healing through IL-10 secretion [85]. Macrophages further contribute to this process by secreting Bone Morphogenic Protein-2 and 4 (BMP2/4) and TGF- β 1, which activate osteoblasts and B/T lymphocytes, reinforcing osteochondral dysfunction [85].

3.2.6. OA as a whole-joint disease

At both the macroscopic and microscopic levels, the joint must be viewed as a functional unit composed of multiple interacting tissues, including cartilage, subchondral bone, synovium, fat pad, muscles, ligaments, and entheses. These elements do not function in isolation but rather engage in continuous mechanical and biochemical communication.

The synovial membrane itself serves as an intermediary within this network. It can be directly activated by breakdown products from cartilage and subchondral bone, including hyaluronan, as well as by cytokines and DAMPs released by BMLs. Once activated, synovial cells release pro-inflammatory cytokines and growth factors such as IL-1, IL-6, and TNF- α , promoting chondrocyte apoptosis and matrix degradation (Figure 5) [5].

Both the synovium and subchondral bone (see above) have been involved as mechanosensitive tissues within the joint. Under abnormal mechanical loading, the synovium can drive osteoclastic bone resorption, contributing to the development of BMLs. Conversely, mechanically stressed subchondral bone (illustrated by BMLs) may activate the overlying

synovium, promoting pannus-like synovial inflammation (Figure 5) [5]. Evidence from patients undergoing meniscectomy shows that mechanical stress can trigger synovitis even in the absence of radiographic OA, underscoring the early involvement of synovitis and the complex crosstalk between joint compartments in OA pathogenesis [97].

The close interconnectivity between articular cartilage and the underlying subchondral bone has been previously discussed.

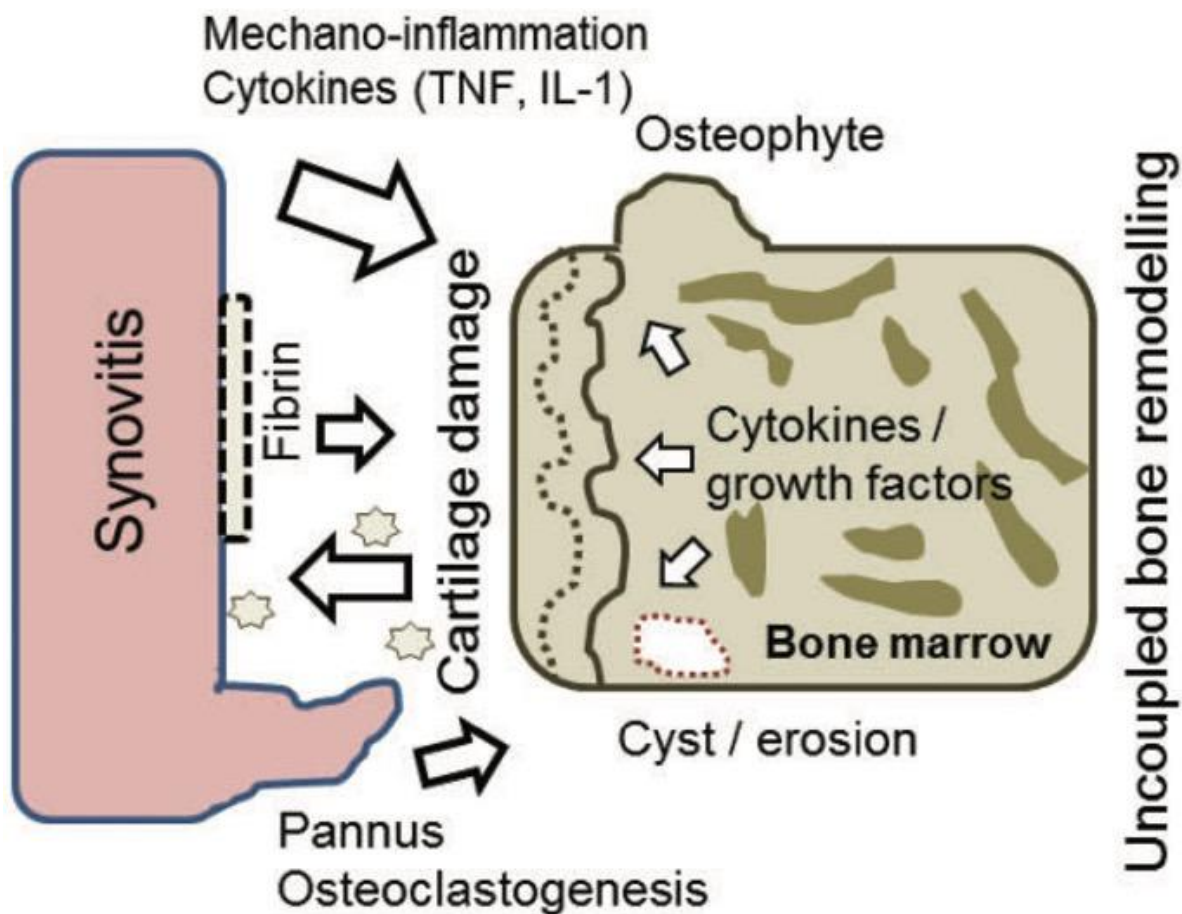


Figure 5. Interplay between synovium, cartilage, and subchondral bone in osteoarthritis pathophysiology. Mechanical stress and inflammatory mediators (e.g., TNF, IL-1) contribute to synovial activation, leading to the release of inflammatory cytokines and the formation of pannus. This promotes cartilage degradation and osteoclastogenesis at the bone interface. Simultaneously, the subchondral bone and bone marrow respond by releasing cytokines and growth factors, further amplifying synovitis and contributing to cyst formation, bone erosion, and uncoupled bone remodeling. These bidirectional interactions between joint compartments fuel the progression of OA [5]. OA: osteoarthritis

Another structure of growing interest in knee OA pathophysiology is the IFP. Functionally, the IFP serves as a mechanical shock absorber and facilitates the distribution of synovial fluid [98].

Anatomically and biologically, it is closely associated with the synovial membrane, forming a distinct functional unit within the joint. Both the IFP and synovium are subjected to parallel inflammatory and fibrotic changes during OA progression [99], and their alterations observed by MRI have been correlated with the increase of inflammation, fibrosis, and vascularization within the synovial compartment [98]. The presence of shared mesenchymal progenitor cells, which can differentiate into myofibroblasts and contribute to tissue remodeling and fibrogenesis, further supports this functional unit [99]. The IFP also directly influences synovial biology: it activates FLS, promoting the expression of extracellular matrix proteins such as collagen and thus driving fibrotic remodeling of the synovial lining [98]. Beyond this local crosstalk, intra-articular adipose tissue, including the IFP, acts as a source of soluble factors and extracellular vesicles [98]. These mediators can directly affect the cartilage, inducing chondrocyte phenotypic shifts toward a catabolic profile and contributing to matrix degradation [98].

Radiographic associations between enthesitis and synovitis were first proposed by McGonagle *et al.* in the context of psoriatic arthritis (PsA), highlighting a shared inflammatory response across both tissues [100]. The same authors later extended this concept to OA, involving enthesitis in OA pathogenesis [71]. However, the causal relationships underlying this association remain to be fully elucidated.

3.2.7. Imaging

Radiographic alterations are typically assessed using two scoring systems. Firstly, radiographic OA has historically been defined using the Kellgren–Lawrence (K–L) grading scale, in which a score of 2 or higher indicates definite OA (Figure 6). However, this classification is now considered relatively coarse and has been progressively complemented by more refined systems. Secondly, the Osteoarthritis Research Society International (OARSI) scoring system (based on images atlas) evaluates specific compartmental features individually, providing a more detailed grading of each structural component [101].

Kellgren-Lawrence score is defined as follow:

- Grade 0: absence of radiological signs of OA.
- Grade 1: possible JSN and minor osteophyte formation (white arrows).
- Grade 2: clearly visible osteophytes (white arrows) and possible JSN.

- Grade 3: osteophyte formation (white arrows) with definite JSN (white triangles), accompanied by some subchondral bone sclerosis and possible bone end deformity.
- Grade 4: large osteophytes (white arrows), pronounced JSN with complete obliteration (black arrows), severe subchondral sclerosis, and clear deformity of the articular surfaces.

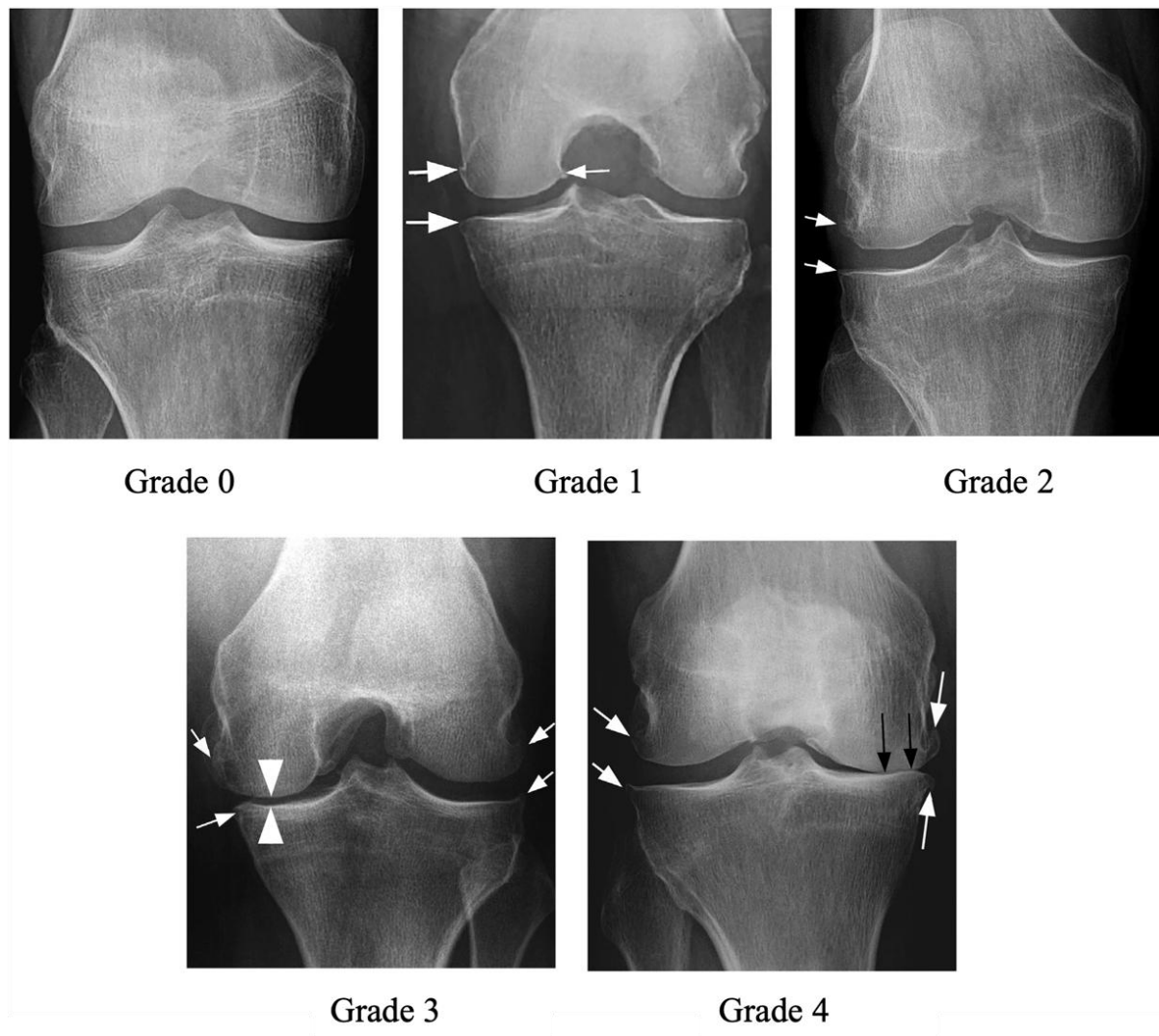


Figure 6. Radiographic grading of knee OA according to the Kellgren-Lawrence scale. Representative anteroposterior knee radiographs illustrating different grades of OA [101].

OARSI score is defined as follow:

- Grade 0–3 (marginal osteophytes): assessed separately at the medial femoral condyle, medial tibial plateau, lateral femoral condyle, and lateral tibial plateau.

- Grade 0–3 (JSN): evaluated in the medial and lateral compartments.
- Present/absent (other features): medial tibial attrition, medial tibial sclerosis, lateral femoral sclerosis

Magnetic resonance imaging (MRI) allows the detection of early alterations in bone and cartilage, as well as architectural changes in other joint tissues, particularly soft tissues, that are not visible on conventional radiography. Several studies have suggested that MRI findings may serve as imaging biomarkers of radiographic OA progression [102–105]. Longitudinal data support synovitis, detected by MRI, as a predictive imaging marker of knee OA progression, underscoring the central role of the synovium, our tissue of interest [102,103]. In the MOST study, Felson *et al.* demonstrated that synovitis was the only structural feature that remained independently associated with incident radiographic OA after adjustment for other joint abnormalities, whereas cartilage damage or cartilage loss did not show independent predictive value [103]. Synovitis is also part of a broader pattern of early pre-radiographic structural changes, including alterations of the IFP [106], degenerative changes in the cruciate ligaments [106,107], meniscal pathology [106], and changes in medial femoral cartilage thickness, which together characterize knees at risk of accelerated OA development [106]. Poor movement patterns or neuromuscular control due to effusion-synovitis, as well as degenerative cruciate ligament instability, may further amplify meniscal damage and joint effusions, highlighting that these tissues are not isolated entities but interact dynamically within the joint environment [108]. These interactions occur on at least a macroscopic scale but also involve biochemical crosstalk through mediators such as cytokines, that play a role in OA [5,8].

Ultrasound also enables the assessment of soft tissue changes, particularly synovial hypertrophy and joint effusion, and has been proposed as a complementary imaging tool in OA [105]. Grey-scale synovitis detected by ultrasound has been associated with pain severity (AUSCAN score) and joint tenderness, and both grey-scale synovitis and power Doppler signal have been linked to radiographic progression, including central bone erosions, osteophyte formation, and joint space narrowing [105]. Notably, in interphalangeal joint OA, the Ghent group led by Wittoek demonstrated that ultrasound is capable of detecting erosions during radiographically non-erosive phases and showed that synovitis was more frequently present in the fully erosive phase compared with non-erosive stages [109]. Furthermore, regarding osteophyte detection, ultrasound is more sensitive than conventional radiography for identifying osteophytes in hand OA [110].

In addition, less conventional imaging and analytical approaches are currently emerging. Spatial transcriptomics and proteomics, operating at a microscopic resolution, enable the identification of cell–microenvironment interactions [111]. A study recently developed a deep learning–based multimodal clustering model that integrates matched multi-omic data from plasma, synovial fluid, and urine to identify biological endotypes of knee OA and predict post-arthroplasty responses [74]. Organoid models, which recreate three-dimensional joint tissue architecture *in vitro*, represent another promising tool to investigate joint inflammation [112]. Finally, deep learning–based artificial intelligence not only allows the detection of lesions that are invisible on standard radiographs but also has the capacity to integrate multimodal imaging and omics data, offering new insights into the complex pathophysiology of OA [113,114].

3.2.8. Treatments

OA significantly impairs quality of life through chronic pain, stiffness, and functional limitations. International guidelines (EULAR, ACR, OARSI) recommend non-pharmacological interventions as first-line therapy, emphasizing a patient-centered approach that includes education, physical activity, weight management, behavioral strategies, and assistive devices. Additional modalities such as heat, cold, acupuncture, or radiofrequency ablation may provide symptom relief in selected patients (Table 2) [115–117].

Non-pharmacological treatments
Core strategies
Exercise (strength, aerobic, flexibility, neuromotor, balance, Tai Chi, Yoga)
Weight loss when appropriate
Patient education and self-management programs
Behavior change techniques (to support physical activity, adherence, etc.)
Cognitive Behavioral Therapy (CBT)
Individualized care plan with progressive adjustment
Assistive and supportive interventions
Bracing (e.g., tibiofemoral knee brace, patellofemoral brace, hand orthoses)
Walking aids (e.g., cane)
Kinesiotaping
Paraffin therapy
Therapeutic footwear, orthotics, workplace adaptations
Symptom relief and adjunctive therapies
Heat and cold therapy
Acupuncture
RFA (radiofrequency ablation – more experimental or reserved for selected patients)

Table 2. Non-pharmacological treatments of OA; adapted from [115,116]

- **Exercise:** Exercise is strongly recommended for knee and hip OA, with robust evidence supporting walking, cycling, neuromuscular training, aquatic and balance, and Tai Chi or yoga. The most effective program is one tailored to the patient's abilities and preferences, as adherence is key to benefit [115,116]. In hand OA, evidence is more heterogeneous. French recommendations propose standardized, self-administered home-based programs including joint mobilization, strengthening, and grip exercises, which improve function and reduce stiffness [118].
- **Weight loss** has a dose–response benefit in OA, improving pain, function, and even systemic inflammation [119–121], with benefits observed after bariatric surgery [122]. Weight loss may also reduce synovitis and slow cartilage loss [123]. However, BMI alone is insufficient, as preserving lean mass is crucial to avoid worsening joint instability [124,125]. In this context, pharmacological approaches such as semaglutide (GLP-1 analog) have shown significant benefits in knee OA in patients with obesity, leading to greater weight loss and reductions in WOMAC pain scores compared to placebo, along with improved physical function [126].
- **Psychosocial and behavioral strategies** combining education, exercise, diet, and self-management provide greater improvements in pain and function than isolated interventions. Multidisciplinary and group-based programs show consistent, although modest, benefits, and cognitive behavioral therapy can further reduce pain and improve mood and quality of life [115,116].
- **Assistance:** the use of canes and tibiofemoral knee braces, as well as first carpometacarpal orthoses is strongly recommended [116,118]. In hand OA, custom rigid night splints (including first metacarpopheal) mainly reduce pain rather than prevent deformity [118]. Other hand orthoses are conditionally recommended, while kinesiotaping remains controversial across guidelines [116,118]. Biomechanical footwear may also improve pain and function in knee OA [127].
- **Symptom relief:** thermal interventions such as hot or cold packs, moist heat, diathermy (electrically delivered heat), ultrasound, paraffin therapy, and radiofrequency ablation are conditionally recommended for the management of OA [116].

Pharmacological treatments are only considered when non-pharmacological approaches fail to provide sufficient symptom relief. They remain to date only symptomatic and do not target the pathophysiology of OA (Table 3). Recommendations are categorized according to their strength and level of evidence. “Strongly recommended” and “conditionally recommended” treatments are supported by clinical evidence and may be used depending on the clinical context and patient profile. In contrast, “conditionally recommended against” refers to interventions with limited or inconsistent efficacy, which are generally discouraged but may still be considered in selected situations. “Strongly recommended against” indicates treatments that are not advised based on current evidence, due to lack of efficacy, absence of clinically meaningful benefit, or an unfavorable benefit–risk balance, and therefore should not be used in routine clinical practice. Importantly, “not recommended” differs from “recommended against”, as it generally reflects insufficient or inconclusive evidence to support the use of an intervention, rather than clear evidence of lack of efficacy or harm.

Pharmacological treatments
Strongly recommended
Topical and oral NSAIDs (non selective and COX-2 inhibitors), ultrasound guided I-A corticosteroid injection
Conditionnally recommended
Acetaminophen (paracetamol), tramadol, topical capsaicin, I-A corticosteroid injection
Conditionnally recommended against
Non-tramadol opioids, intra-articular hyaluronic acid (IAHA), colchicine, prolotherapy
Strongly recommended against
Bisphosphonates, glucosamine, chondroitin sulfate, HCQ, methotrexate, SCI, PRP treatment, biologics

Table 3. Pharmacological treatments of OA. NSAIDs: nonsteroidal anti-inflammatory drugs; COX-2: cyclooxygenase 2; I-A: intra-articular; HCQ: hydroxychloroquine; SCI: stem cell injection; PRP: platelet rich plasma; OA: osteoarthritis; adapted from [116,117].

- Nonsteroidal anti-inflammatory drugs (NSAIDs): topical NSAIDs are strongly recommended as first-line therapy for OA pain due to lower systemic exposure, although they are less effective for deep joints such as the hip. Oral NSAIDs are more controversial: while the ACR supports their use, OARSI recommends them conditionally, preferably with a proton pump inhibitor (PPI) or selecting cyclooxygenase 2 (COX-2) inhibitors to reduce gastrointestinal risks [116,117]. They are contraindicated in patients with cardiovascular comorbidities or frailty [128,129], and French guidelines restrict their use to short-term management of inflammatory flares at the lowest effective dose [118].

- Oral corticosteroids: similarly to oral NSAIDs, systemic corticosteroids should be reserved for inflammatory flare-ups in hand OA, used at the minimal effective dose and for short durations, particularly in polyarticular forms, and are not recommended in knee OA [118].
- Intra-articular injections: glucocorticoid injections are recommended for knee and hip OA, ideally under ultrasound guidance, but in hand OA they should be limited to interphalangeal joints during inflammatory flares; thumb base injections are not supported by randomized controlled trial (RCT) evidence [116–118]. In contrast, hyaluronic acid (HA) injections are conditionally recommended for longer-term relief, with effects lasting beyond 12 weeks and a favorable safety profile [116,117]. Current consensus suggests reserving viscosupplementation for selected patients based on symptom severity, comorbidities, lack of alternatives, or refusal of arthroplasty [130]. Prolotherapy (irritant solution to stimulate tissue repair) [116], stem cell [131] and platelet rich plasma injections [118] are not recommended.
- Oral analgesic: acetaminophen and tramadol may be considered when NSAIDs are contraindicated, although acetaminophen has limited efficacy and tramadol lacks long-term safety data; in hand OA, French guidelines do not recommend tramadol due to limited benefit and risk of dependence [118]. Duloxetine may be preferable, particularly in patients with depressive symptoms [116,117]. Non-tramadol opioids and colchicine are not supported, and bisphosphonates, glucosamine, and hydroxychloroquine (HCQ) have shown no meaningful benefit [116,118]. Chondroitin sulfate is not recommended for knee or hip OA [116] but may be considered in hand OA for pain relief only [118]. A recent meta-analysis states that curcumin is effective in reducing pain and joint stiffness [132]. Regarding micronutrients, the antioxidant role of vitamins E, C, and A, as well as selenium, has not been definitively established [133]. Vitamin D represents a promising therapeutic intervention in OA, as deficiency is associated with increased inflammation and cellular senescence [134], while supplementation may improve muscle strength and exert protective metabolic and anti-inflammatory effects [52,62,135].
- Disease-modifying agents: methotrexate (20 mg/week) showed moderate pain reduction in hand OA at 6 months in one RCT [136], whereas TNF and IL-1 inhibitors failed to

demonstrate efficacy for pain [116,137–140]. However, etanercept was associated with radiographic remodeling and reduced bone marrow lesions at one year [139]. Nerve Growth Factor (NGF) inhibitors have been shown to effectively reduce pain and improve mobility in patients with OA, but, their clinical use has been limited due to significant adverse effects, including rapidly progressive OA, chondrolysis, and bone destruction, which ultimately led to safety concerns and withdrawal from development programs [141–143]. However, more recent data from an ACR abstract reporting a Phase II study of LEVI-04, a novel neurotrophin-3 inhibitor, showed no increased incidence of serious adverse events, including rapidly progressive OA[144].

- Surgery: osteotomy should be prioritized in young adults with symptomatic hip or knee OA, especially when underlying structural issues such as dysplasia or varus/valgus deformities are present. In cases where symptoms persist despite optimal conservative management and radiographic evidence confirms advanced OA, joint replacement surgery may then be considered to alleviate pain and restore function [145].
- Novel therapies: genicular artery embolisation has emerged as a potential therapy for managing knee OA pain, as demonstrated in a recent RCT versus sham [146]. This minimally invasive procedure reduces inflammation and pain by blocking abnormal synovial blood vessels. A new trial is currently underway comparing this approach with intra-articular corticosteroid injections [147]. In the ESTIVAL trial, a French randomized, sham-controlled, multicentre, double-blind study of transcutaneous auricular vagus nerve stimulation, a non-invasive neuromodulation technique that stimulates the auricular branch of the vagus nerve to modulate inflammatory and pain pathways, in erosive hand OA, the primary endpoint of VAS pain reduction was not met. However, improvements were observed in functional outcomes and patient global assessment, with greater VAS pain reduction in patients presenting higher baseline joint inflammation [148]. Consistent with these findings, a 12-week transcutaneous vagus nerve stimulation protocol targeting the auricular branch of the vagus nerve in knee OA showed a significant reduction in VAS pain scores [149]. Several phase 3 trials are currently investigating novel disease-modifying approaches for OA. Lorecivivint (SM04690), a Wnt/ β -catenin pathway inhibitor, has shown potential in pain reduction [150]. TissueGene-C is an intra-articular gene and cell therapy designed to locally produce TGF- β , aiming to promote anabolic cartilage repair [151], while cindunistat

(SD-6010), an iNOS inhibitor, targets oxidative stress to limit cartilage degradation [152]. Anabolic strategies include LNA043 (an ANGPTL3 derivative), which promotes chondrogenic differentiation via integrin $\alpha 5 \beta 1$ and is currently in phase 2b development, and sprifermin (FGF18), which enhances chondrocyte activity and cartilage thickness in phase 2 trials [153,154]. Liposomes are also being explored, both as intra-articular drug carriers with prolonged retention and for their intrinsic anti-inflammatory and lubricating properties, potentially reducing cartilage damage [155,156].

- Others: in a randomized placebo-controlled phase 2a trial, denosumab, a monoclonal antibody targeting RANKL and inhibiting osteoclast-mediated bone resorption, demonstrated structure-modifying effects in erosive hand OA by inducing remodeling of existing lesions and preventing the development of new erosive joints [157]. In recent RCTs, metabolic agents have been evaluated in knee OA. In the trial by Pan et al., metformin significantly reduced knee pain compared with placebo, and this effect remained significant after adjustment for weight loss. Beyond its well-known metabolic effects, metformin may also reduce inflammation, oxidative stress, and pain through activation of 5'-adenosine monophosphate-activated protein kinase (AMPK). These mechanisms may partly explain its beneficial effect on knee pain in OA [158]. Once-weekly semaglutide 2.4 mg also resulted in a significant reduction in knee pain among individuals with obesity [159]. Intra-articular GLP-1 and its analogues may exert local anti-inflammatory effects on joint tissues by modulating cytokine expression through GLP-1 receptors expressed on chondrocytes and FLS, while promoting chondrocyte survival and autophagy. In addition, they may locally reduce cartilage degradation by decreasing matrix metalloproteinase expression, and a phase 2 clinical trial is currently ongoing [160].

3.3. Synovitis

Since OA is now seen as a whole-joint disease and not only a cartilage degeneration, synovitis is considered as a key contributor to disease onset and progression [1,8]. This research will therefore focus on the synovial membrane and the mechanisms driving synovitis.

3.3.1. Anatomy and histology

The synovial membrane is a specialized connective tissue that lines the inner surface of the joint capsule and surrounds the articular cavity, covering all intra-articular, non-cartilaginous structures (Figure 7). It plays a key role in maintaining joint homeostasis and lubrication [1,161].

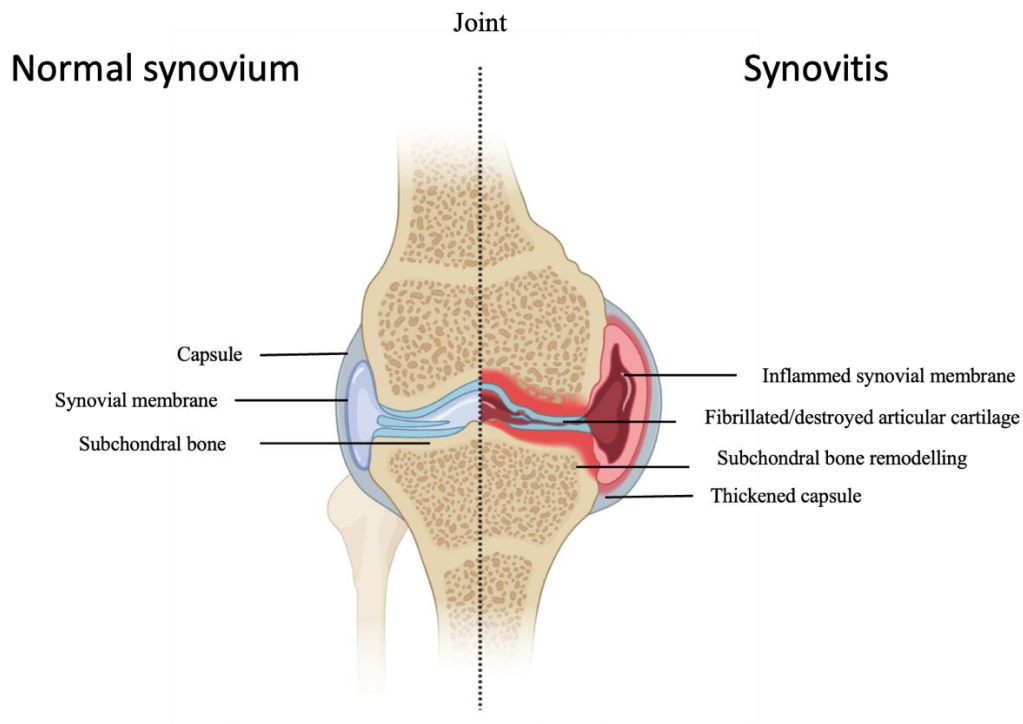


Figure 7. Anatomy of the synovial membrane; adapted from bioRender (Illustration software).

Normal synovium consists of two distinct layers: the lining layer and the sublining layer. The thin lining layer is in direct contact with the synovial fluid. This layer contains two main types of synoviocytes: i) macrophage-like synoviocytes (MLS), which represent about 75% of the lining cells and are involved in phagocytosis, and ii) FLS, which produce key components of the synovial fluid, including hyaluronan and lubricin. The sublining is more loosely organized and relatively paucicellular, consisting mainly of blood vessels, fibroblasts, and adipocytes, along with a small number of resident interstitial macrophages and lymphocytes embedded in a collagen-rich extracellular matrix (Figure 8) [161]. MLS contribute to the formation of an immunological barrier through tight junctions, thereby helping limit the spread of inflammation within the synovial membrane [162].

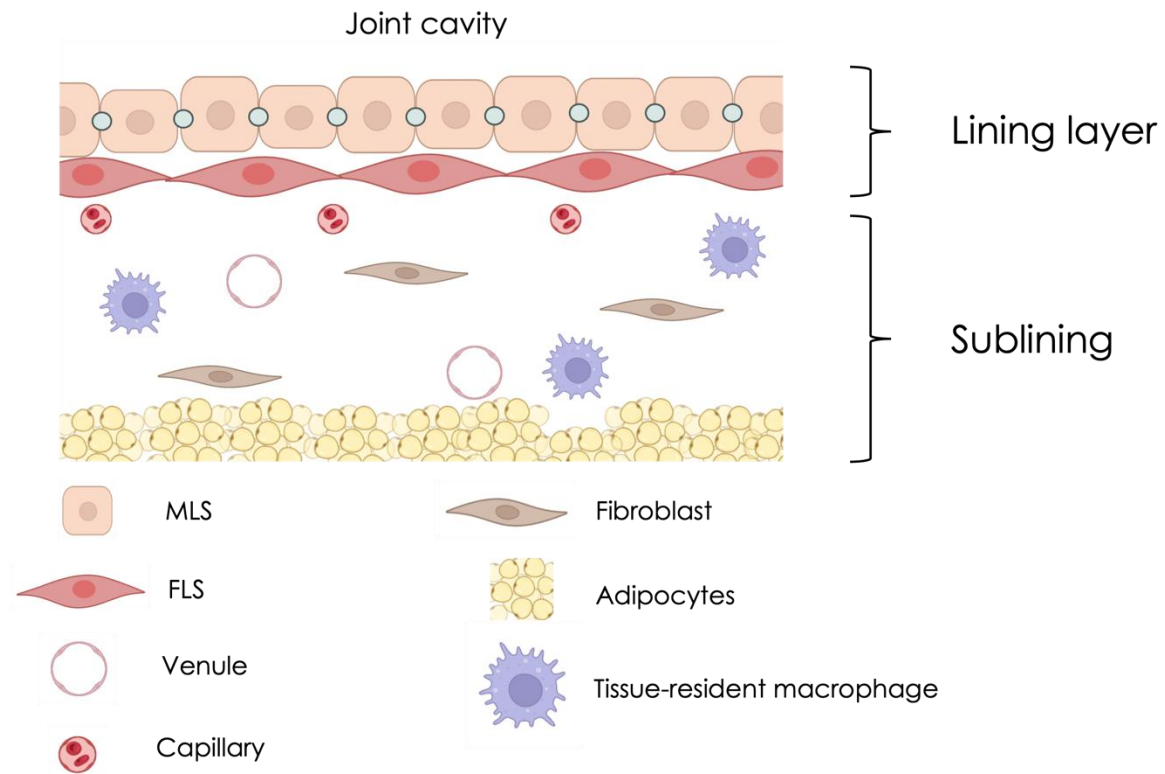


Figure 8. Illustrative schema of synovial membrane histology; created with bioRender. MLS: macrophage-like synoviocytes; FLS: fibroblast-like synoviocytes.

Synovial inflammation, or synovitis, is primarily characterized by infiltration of inflammatory cells, formation of new blood vessels (neoangiogenesis), sublining fibrosis and lining cell proliferation (hyperplasia) (Figure 9) [1,8].

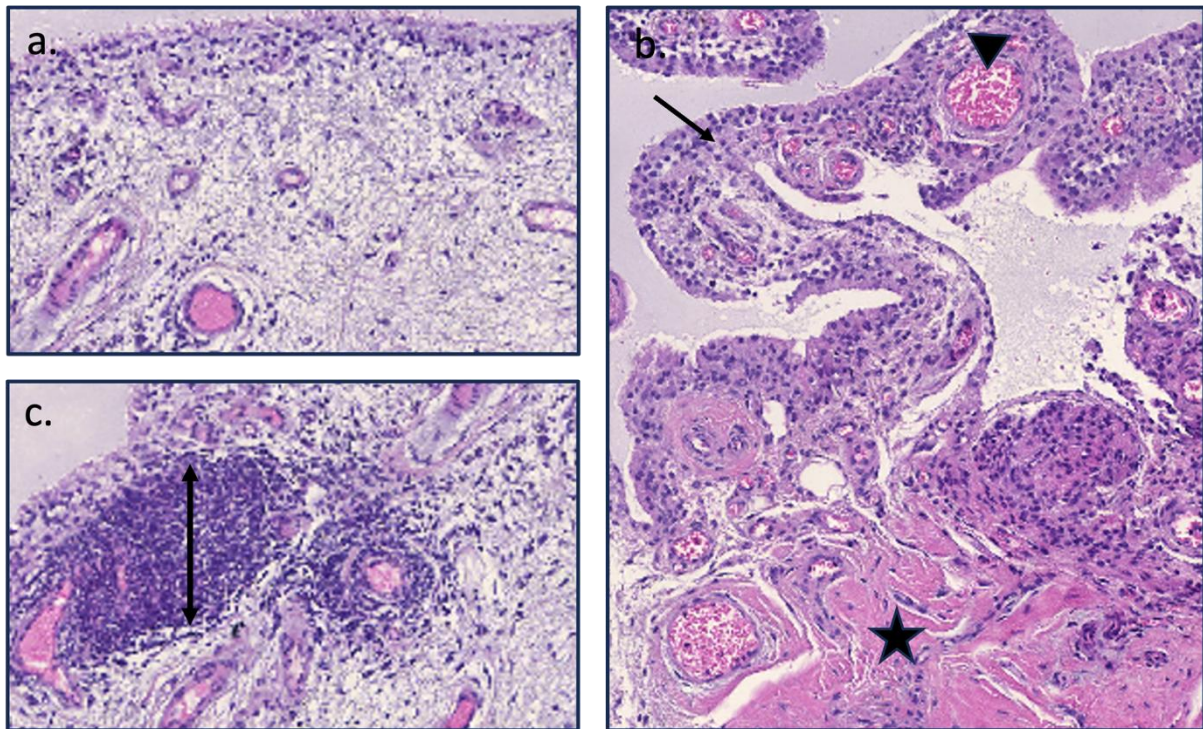


Figure 9. H&E staining of synovial membrane a) non-inflamed OA synovium; inflamed OA synovial membrane with b) hyperplasia (arrow), neovascularization (arrowhead) and fibrosis (star) and c) leukocytes infiltrates (double-headed arrow). 20x. H&E: hematoxylin & eosin from laboratory.

3.3.2. Leukocytes infiltration, neoangiogenesis, fibrosis and hyperplasia are hallmarks of synovitis

Leukocyte infiltration in OA is primarily characterized by macrophages, though their infiltration is less pronounced than in RA [163]. Debris within the joint cavity, including matrix fragments released from cartilage, subchondral bone or BML, act as DAMPs [1,8]. These molecules bind to Toll-like receptors (TLR 2 and TLR4) on synovial cells, thereby triggering the activation of macrophages and subsequent NF- κ B signaling pathway [164]. Consequently, activated macrophages produce pro-inflammatory cytokines and chemokines, such as TNF- α and IL-1 β , as well as matrix MMPs. These mediators promote the recruitment of additional immune cells, including macrophages, granulocytes, and lymphocytes. They also directly contribute to cartilage degradation and FLS proliferation. In addition, pro-inflammatory cytokines further amplify inflammation by inducing their own production and by stimulating synovial cells and chondrocytes to release IL-6 and IL-8 [8,163,165]. The resulting cytokine signature correlates with symptom severity in OA patients, possibly by modulating cytokine-mediated nociception [97,166]. The activation and recruitment of inflammatory cells can also be driven by the complement cascade. Its components may originate from plasma, but also be

locally produced by chondrocytes or macrophages, contributing to the amplification of synovial inflammation (Figure 10) [8]. Conversely, macrophages can also adopt a resident/anti-inflammatory phenotype. In this context, they contribute to the resolution of inflammation [167], partly through the secretion of anti-inflammatory cytokines such as IL-4, IL-10, and IL-13 [1].

Early studies showed that mast cell infiltration in OA synovium correlates with synovitis severity (Krenn score) and radiographic progression (KL grade) [168]. Mechanistically, mast cells contribute to synovial inflammation through the release of mediators such as histamine, tryptase, and pro-inflammatory cytokines, promoting FLS activation, leukocyte recruitment, angiogenesis, and cartilage degradation [169,170]. Beyond this association, experimental studies demonstrate a causal role, as mast cell activation, particularly via tryptase and IgE/FcεRI signaling, drives inflammation and tissue damage, while their deficiency or inhibition attenuates OA features [169].

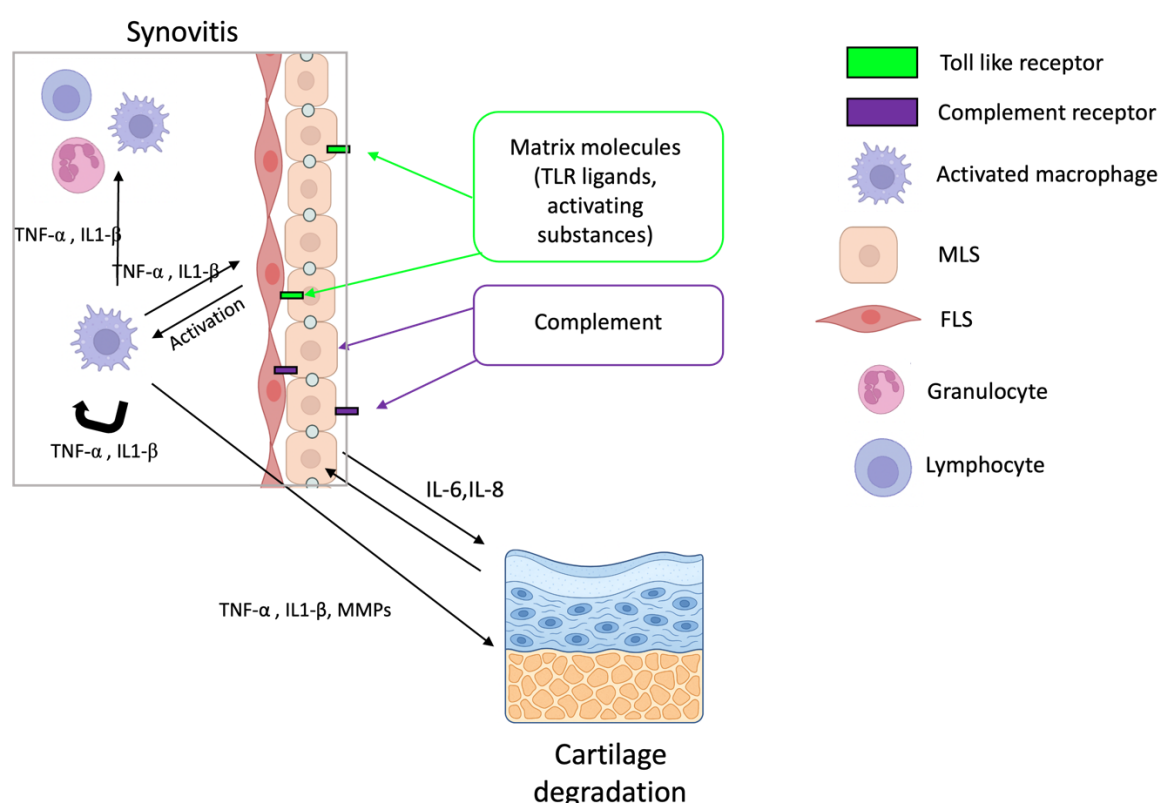


Figure 10. Mechanisms of leukocyte infiltration within the synovium. Cartilage, bone, or meniscal debris act as DAMPs that engage TLR2/4 on synovial cells, activating NF-κB signaling. This induces pro-inflammatory cytokines (TNF-α, IL-1β), chemokines, and MMPs, which recruit additional macrophages, granulocytes, and lymphocytes, drive cartilage degradation and induce FLS proliferation. These cytokines amplify inflammation by reinforcing their own production and by stimulating synovial

cells and chondrocytes to release IL-6 and IL-8. TNF: tumor necrosis factor; IL1: Interleukine 1; MLS: macrophage-like synoviocytes; FLS: fibroblast-like synoviocytes. Adapted from [8].

Several studies also highlighted the key role of adaptive immunity in OA. A CD4⁺/CD8⁺ cell ratio of approximately 5:1 has been reported, indicating a substantial T-cell presence [171]. Multiple CD4⁺ T-cell subsets have been identified, including Th1, Th2, Th17, and regulatory T cells such as Th3 and Tr1 [172]. These T cells contribute to synovial inflammation through the secretion of pro-inflammatory cytokines. TNF- α and IL-6 promote inflammatory signaling [166], while IL-17 supports neutrophil recruitment and induces the production of additional pro-inflammatory cytokines and chemokines. IL-22 further amplifies inflammation by enhancing innate immune responses [173]. B cells have also been detected in OA synovium and may further modulate the local immune environment [173]. In addition, neutrophils can be detected in the synovial membrane during synovitis, albeit to a lesser extent than in RA, and are less abundant than macrophages and T cells [173,174].

Synovial neovascularization, predominantly localized in the sublining compartment, not only facilitates immune cell infiltration, partly via endothelial adhesion molecules like E-selectin, thereby amplifying inflammation, but also supplies (such as the subchondral bone) nutrients to avascular cartilage tissue [16,175]. This process may contribute to the transition from acute to chronic inflammation [175]. It results from an imbalance between pro-angiogenic (VEGF and hypoxia-inducible factor-1 α), and anti-angiogenic signals [175–177]. Thus, angiogenesis can drive inflammation, just as inflammation promotes further angiogenesis [175].

Synovial hyperplasia is caused by the proliferation of FLS [178]. In RA, inflammation induces rapid structural changes in the synovial lining, including alterations in lining thickness and MLS organization, leading to the loss of normal cell–cell contacts with further activation of FLS [162]. RNA-sequencing studies have highlighted distinct functional states of FLS depending on their anatomical localization and disease context. In RA, human and murine models have shown that lining FLS are primarily involved in synovial interface and homeostatic functions, including synovial fluid production and tissue maintenance, but can also acquire migratory properties during the course of arthritis [179,180]. In contrast, sublining FLS adopt a pro-inflammatory and stromal phenotype across both murine models and human synovium, characterized by cytokine production, extracellular matrix remodeling, fibrotic pathway activation, and enrichment in cytokine-response programs [179,180]. In murine models,

transcriptional analyses further indicate enrichment of TGF- β /SMAD pathway components, consistent with fibrotic stromal responses (see below) [179]. In OA, human studies suggest that inflammatory activity is more restricted and may be driven by specific sublining fibroblast subsets, notably CD90⁺ populations. However, the exact contribution of CD34-defined subsets remains debated, as some studies report higher IL-6 production in CD90⁺CD34⁺ fibroblasts [181], whereas others associate pro-inflammatory cytokine and chemokine production predominantly with CD90⁺CD34⁻ FLS [182,183]. In physiological condition, synovial membrane acts as a filter between blood vessels and joint fluid, keeping large protective molecules like hyaluronic acid and lubricin inside the joint, while blocking plasma proteins [8]. When inflammation occurs, this barrier breaks down, allowing these protective molecules to leak out [8,184,185].

The p38/JNK/MAPK/ERK1/2 and PI3K/AKT signaling pathways play a central role in the regulation of cell proliferation across multiple cell types, including fibroblasts and cancer cells [186–188]. These pathways are primarily activated by TGF- β within the tissue microenvironment, as demonstrated in primary human lung fibroblasts [189] and cancer cell models [190]. Importantly, their role in promoting proliferation has also been specifically demonstrated in FLS [191]. More specifically, in OA FLS, pharmacological inhibition of the MAPK/ERK and PI3K/AKT pathways has been shown to significantly reduce cell proliferation [192,193] (Figure 11).

Fibrosis is characterized by excessive deposition of extracellular matrix components, including collagen and fibronectin, produced by activated fibroblasts, leading to progressive thickening and stiffening of the synovial membrane, as reported in human synovial tissue in the context of hip dysplasia [194]. In contrast to inflammation, which may occur early in the disease course, fibrosis typically emerges in late-stage OA [195]. Although TGF- β activates multiple interconnected pathways, including SMAD2/3/4, p38/JNK/MAPK, RhoA-ROCK, and PI3K/AKT signaling, the fibrotic program is predominantly governed by canonical SMAD2/3/4 and RhoA-ROCK activation, as broadly described across cell types in the literature [196] and specifically illustrated in FLS (Figure 11) [191].

Importantly, the balance between ALK5–Smad2/3/4 and ALK1–Smad1/5/8 signaling determines context-dependent fibrotic outcomes in FLS, influencing the extent and nature of synovial remodeling [191].

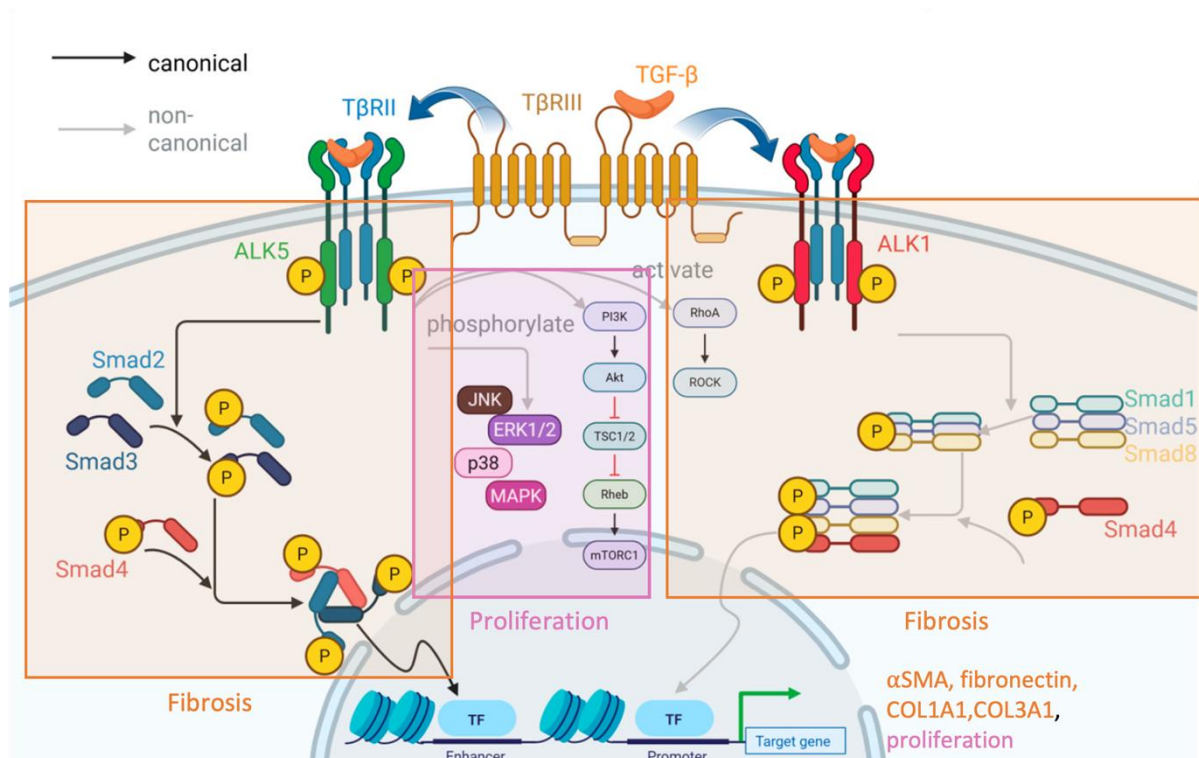


Figure 11. TGF- β signaling driving proliferation and fibrosis in OA. TGF- β binds to T β RII on synovial fibroblasts, a process facilitated by the co-receptor T β RIII, which then activates type I receptors. In the canonical cascade, ALK5 phosphorylates Smad2 and Smad3, which associate with Smad4 and move into the nucleus to regulate transcription of pro-fibrotic genes (α SMA, fibronectin, COL1A1, COL3A1). Non-canonical signaling is also triggered, involving MAPKs (p38, JNK, ERK1/2), PI3K–AKT–mTOR, and RhoA–ROCK pathways. In contrast, ALK1 drives phosphorylation of Smad1, Smad5, and Smad8, which form complexes with Smad4, translocate to the nucleus to influence distinct target gene expression. Canonical SMAD2/3/4 and RhoA–ROCK pathways primarily regulate extracellular matrix production and fibrotic activation of FLS (orange), while p38/JNK/MAPK and PI3K/AKT signaling mainly support cell proliferation (pink). OA: osteoarthritis; adapted from [191].

TGF- β not only induces fibroblast proliferation [197] and ECM deposition [195], but also attracts immune cells like leukocytes and contributes to osteophyte formation [195]. TGF- β has been associated with the expression of epithelial-to-mesenchymal transition (EMT) genes (e.g. cadherins) in OA FLS, suggesting partial EMT-like features, as the synovial lining layer is not an epithelium per se [198,199]. Our laboratory identified the EMT pathway as significantly downregulated in human OA FLS following CEMIP or Janus kinase depletion/inhibition [200,201]. This finding more likely reflects modulation of fibroblast-to-myofibroblast transition (FMT), characterized by increased α -SMA and fibronectin expression, rather than true EMT [200,201]. In this context, RA FLS acquire invasive motility and actively contribute to extracellular matrix deposition [202]. In addition, myofibroblasts induce a destructive phenotype in co-cultured OA chondrocytes, leading to increased secretion of MMPs [199].

3.3.3. CD55 (lining) FLS and CD34 (sublining) FLS

Recent advances in single-cell and bulk RNA sequencing have enabled the characterization of distinct FLS subtypes based on their gene expression profiles. FLS in the lining layer of RA are involved in the production of synovial fluid (hyaluronan) and are characterized by the expression of CD55 [180]. CD55 accelerates the decay of C3 convertases, thereby regulating immune system activation and protecting cells from the deleterious effects of complement activation [203]. It may also help prevent the deposition of immune complexes by binding to the collagen-rich fiber network within the RA synovium [203]. In contrast, CD90 marks sublining FLS, which are enriched in matrix MMPs and inflammatory mediators, as shown in human RA synovium [204,205] and confirmed across both RA and OA single-cell RNA-sequencing studies [182]. In human RA, these cells show higher expression of proliferation markers such as Ki67, as well as genes involved in migration and invasiveness, including CTHRC1, TWIST1, and POSTN, suggesting a greater invasive potential [206]. Notably, these genes are more strongly expressed in human RA than in OA [182,207]. Further studies identified CD34 as a novel marker of sublining OA FLS. This marker distinguishes CD90⁺CD34⁺ FLS, which highly express genes associated with fibrosis and angiogenesis, from CD90⁺CD34⁻ FLS, which predominantly produce inflammatory cytokines and chemokines, although these functional distinctions may vary across studies [182,183]. Chen *et al.* demonstrated that a distinct CD34⁺ sublining FLS cluster characterized by high DKK3 expression exhibited the strongest upregulation of the fibrotic TGF- β signaling pathway and showed the capacity to differentiate into myofibroblasts, as observed in human arthrofibrosis following joint trauma or surgery [208]. Consistently, a recent study by Collins *et al.* (2026) reported that sublining FLS expand after joint injury and exhibit regenerative potential, partly through differentiation into myofibroblasts [209]. Interestingly, OA and RA CD90⁺CD34⁺ fibroblasts may exhibit mesenchymal stem cell-like properties, including the potential for osteogenic and chondrogenic differentiation [210].

These two markers will be both useful for our further investigations.

3.3.4. Inflammatory histological scorings

Synovial features are reflected by several inflammatory histological scoring systems. The Tak score (0-19) includes the following graded items: lining layer hyperplasia (0-3), neutrophils (0-4), lymphocytes (0-4), plasmocytes (0-4) infiltration and vascularization (0-4) [211]. However,

it should be noted that the Tak score has not been formally validated in OA. The Krenn synovitis score is a semi-quantitative score ranging from 0 to 9, based on three items: lining layer hyperplasia (0–3), stromal cell density (0–3), and leukocyte infiltration (0–3). The total score distinguishes low-grade from high-grade synovitis [212]. The IMSYC score extends the Krenn score by adding five IHC markers: CD68 (macrophages), CD3 (T cells), CD20 (B cells), CD31 (vascularization), and Ki67 (proliferation), each graded from 0 to 3. This combined approach allows a more detailed characterization of synovial inflammatory activity [213].

In the present study, we chose to use the Tak score despite its lack of formal validation in OA, primarily for reasons of reproducibility and consistency. It had been applied in our initial proteomic study [13] and was routinely used in our clinical pathology practice, facilitating comparison across analyses. Moreover, unlike the Krenn score, the Tak score distinguishes between different leukocyte subtypes, allowing a more nuanced inflammatory profiling and better discrimination between pathologies. In contrast, the Krenn score tended to reach saturation even in non-inflammatory (NIN) OA samples, limiting its discriminatory capacity.

We applied a modified version of the Tak score, that will be referred to as the “Tak score” in the remainder of the manuscript, for clarity and ease of reading. The vascular component was excluded, as vessel density was poorly discriminative between synovitis subtypes and remained elevated even in mildly inflammatory OA samples, rapidly reaching maximal scores (3–4/4) and thus limiting its ability to capture differences across inflammatory grades. Synovial lining hyperplasia was graded on a 0–4 scale instead of 0–3 to better capture the degree of lining cell proliferation, and neutrophil infiltration was graded on a 0–3 scale instead of 0–4, reflecting its generally lower prominence compared with mononuclear cell infiltration. In the initial proteomic study, macrophage infiltration was additionally assessed by CD68 IHC; however, this parameter was not retained in subsequent analyses to simplify the scoring procedure and facilitate its application to larger cohorts.

Fibrosis was not specifically assessed using a dedicated histological scoring system, such as the one recently proposed by Obeidat *et al.* [214], which was developed in DMM (Destabilization of the Medial Meniscus) and PMX (Partial Medial Meniscectomy) models, although it would have been relevant within the scope of synovial remodeling. This approach, which relies on a semi-quantitative and largely morphology-based (binary) assessment of stromal expansion, was

not retained, as it was considered less suited for objective and reproducible comparisons across cohorts.

3.4. Rheumatoid arthritis

Synovitis is also a hallmark of RA and represents the central pathological process driving joint damage in this disease. In contrast to OA-associated synovitis, which is often milder and more heterogeneous, RA synovitis is characterized by a highly organized and persistent inflammatory response, leading to the formation of an invasive pannus that progressively erodes cartilage and bone. Histologically, RA synovitis displays marked lining layer hyperplasia (Figure 12A), dense leukocyte infiltration, prominent neovascularization (Figure 12B), and fibrosis. Due to its robust and well-characterized inflammatory phenotype, RA synovitis will serve in this study as a positive control for synovial inflammation when compared with OA [215]. Unlike OA, which is primarily a localized degenerative condition, RA is a chronic autoimmune and highly inflammatory systemic disease that may also involve extra-articular tissues and organs [216]. It usually appears as a chronic, symmetrical, polyarticular condition that primarily affects the small joints in the hands and feet [216,217]. The 2010 ACR/EULAR classification criteria for RA are based on a score $\geq 6/10$, integrating the number and type of joints involved (with higher weight given to small joint involvement), serological markers [rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPAs)], elevated acute-phase reactants [C-reactive protein (CRP) or erythrocyte sedimentation rate (ESR)], and symptom duration of at least six weeks [218]. Its prevalence reaches up to 0.2% in men and ranges from 0.3% to 0.6% in women in Europe, with an overall prevalence of 0.46% [219,220]. Its development results from a combination of genetic and environmental factors. Up to 66% of genetic susceptibility to RA is linked to specific HLA alleles, particularly HLA-DR4 and HLA-DR1. These alleles, in combination with environmental triggers such as smoking, enhance the presentation of citrullinated self-antigens to autoreactive T cells, thereby promoting the production of ACPAs [221–223]. However, in about 30% of cases, the disease occurs without detectable ACPAs, and the underlying causes remain unclear [221]. At the molecular level, the marked synovial hyperplasia and dense inflammatory infiltrate observed in RA are driven by tightly orchestrated immune-cell recruitment and activation processes. Leukocyte adhesion to the endothelium and subsequent infiltration represent early and critical events in RA, driven by CC and CXC chemokines secreted by activated FLS, MLS, or T cells. Beyond promoting leukocyte recruitment, these chemokines further amplify synovial activation by stimulating FLS proliferation and sustained

cytokine production (e.g., CCL2, CCL5, CXCL8), particularly in response to inflammatory mediators such as TNF- α , IL-1 β , and IFN- γ . This persistent inflammatory signaling promotes synovial hyperplasia and angiogenesis, ultimately leading to the formation of an invasive pannus. The pannus, composed of proliferating FLS, immune cells, and newly formed vessels, directly invades adjacent cartilage and bone, secreting high levels of MMPs and additional pro-inflammatory mediators that drive progressive tissue destruction [204,216,217].

Lymphocytic infiltration can also organize into ectopic lymphoid follicles that present antigen to monocytes, macrophages, dendritic cells, B cells or plasma cells. The latter are the cells responsible for the production of RF or ACPAs (Figure 12C) [223,224].

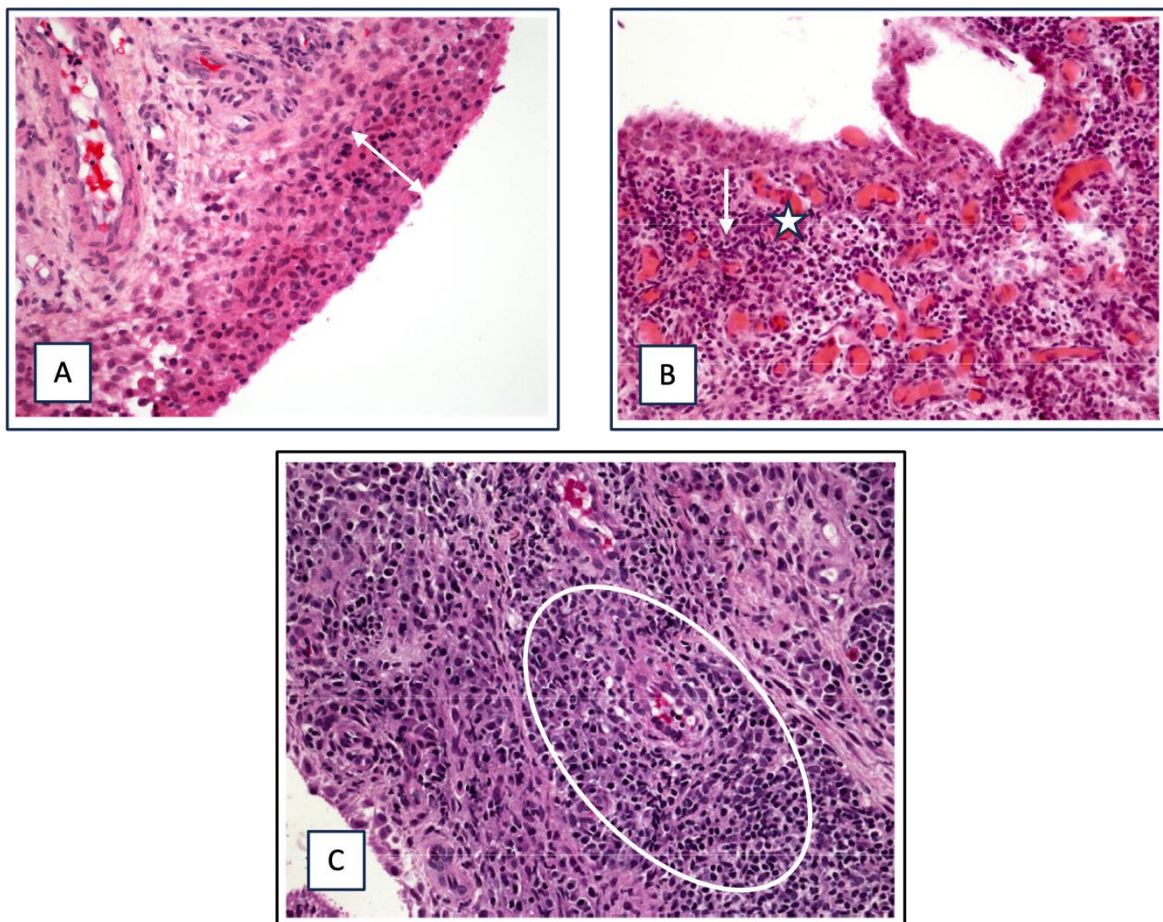


Figure 12. Histopathology of RA synovitis. A) Hyperplasy (doubled-arrow), B) leucocyte infiltration and neoangiogenesis and C) lymphoid follicule from [225]

T cells play a key role in RA, as they receive antigenic stimulation from monocytes, macrophages, dendritic cells, B cells, and plasma cells, as evidenced by the formation of ectopic

lymphoid follicles within the inflamed synovium [223,224]. Among these, two major T helper subsets have been identified: IFN- γ -producing Th1 cells and IL-17-producing Th17 cells. IFN- γ contributes to RA pathophysiology by activating macrophages and upregulating major histocompatibility complex (MHC) class II expression, thereby enhancing antigen presentation. This signaling is dependent on the JAK–STAT pathway, making it susceptible to inhibition by JAK inhibitors. Conversely, IFN- γ -producing CD8⁺ T cells can also secrete the anti-inflammatory cytokine IL-10, highlighting the context-dependent effects of IFN- γ [226]. Th17 cells contribute significantly to inflammation through IL-17, which stimulates the release of pro-inflammatory cytokines such as TNF- α , IL-1 β , and IL-6, thereby amplifying the inflammatory response [227,228]. IL-17 also promotes the production of chemokines that attract neutrophils, macrophages, and lymphocytes into the synovium [223,228]. In addition, Th17 cells release GM-CSF and IL-22, which also support leukocyte recruitment [229].

B cells contribute to RA pathogenesis not only through antigen presentation and autoantibody production, but also by promoting osteoclastogenesis via RANKL expression, thereby exacerbating bone erosion [230]. The ACPAs they generate further amplify inflammation by stimulating immune cells, activating complement pathways, and inducing pro-inflammatory cytokine release [231]. This pro-inflammatory environment is reinforced by NETosis, a process in which neutrophils release DNA-based fibers that both damage tissues and expose citrullinated proteins, thereby perpetuating the autoimmune response [232].

RA is also characterized by the infiltration of M1 macrophages, a classically activated, pro-inflammatory macrophage subset induced by stimuli such as IFN- γ and microbial signals, that promote joint destruction by producing TNF- α and IL-1. In contrast, M2 macrophages, which represent alternatively activated macrophages associated with tissue repair and resolution of inflammation, secrete anti-inflammatory mediators such as IL-10 and TGF- β , and play a role in angiogenesis, tissue remodeling, and repair [223,233]. Notably, the M2/M1 ratio was inversely correlated with immune infiltration, as assessed by the CD45⁺ cell fraction in the synovium by IF [234].

Although OA and RA synovitis share certain inflammatory features, suggesting a form of inflammatory continuum, important differences distinguish the two conditions in terms of distribution, intensity, and structural consequences.

Regarding distribution, synovial inflammation in OA is typically patchy and localized to areas adjacent to cartilage damage and can already be detected in early stages of the disease, even before extensive cartilage loss. It has been associated with structural progression and pain severity. In contrast, RA synovitis is diffuse throughout the joint and reflects a persistent and systemic inflammatory process [175,235].

Concerning lining layer hyperplasia, OA may exhibit synovial thickening, particularly in advanced stages, where its microscopic appearance can resemble that of RA [175,236]. In RA, as the disease progresses, this hyperplastic appearance may become less prominent and give way to a more disorganized synovial architecture, contributing to the formation of an invasive pannus [235]. Huang *et al.* showed that RA FLS exhibit a more sensitive inflammatory phenotype than OA FLS, characterized by increased proliferation, migration and resistance to apoptosis [237].

With respect to leukocyte infiltration, OA synovium contains macrophages and T cells that appear to be activated locally and express adhesion molecules, although less abundantly than in RA. In both RA and OA, higher numbers of leukocytes are found in the sublining compared with the lining compartment. However, in RA, neutrophils are increased approximately fivefold in the sublining compared with the lining layer, a pattern not observed in OA. Eosinophils are detected exclusively in the sublining compartment in both diseases but are more abundant in RA [238]. In contrast, RA synovitis is characterized by dense and organized leukocyte infiltration (nodules), sustaining chronic immune activation, although similar infiltrates can also be observed in OA synovium, generally to a lesser extent [175].

Regarding neovascularization, OA displays vascular remodeling and increased vessel density, but indices of endothelial proliferation are lower. By comparison, RA shows more marked endothelial activation and angiogenesis. Although VEGF levels are typically higher in RA synovial fluid, VEGF expression within synovial tissue appears similar in both diseases [175].

Beyond these histological aspects, FLS also exhibit disease-specific phenotypes. In early OA, synovial tissue from painful regions shows upregulation of pro-fibrotic and pro-inflammatory genes, and both early and end-stage OA demonstrate increased expression of nociceptive signaling pathways and neuronal growth genes [239]. End-stage OA FLS may display a transcriptional profile similar to leukocyte-rich RA FLS, suggesting partial convergence at advanced stages [182]. However, RA FLS generally exhibit a higher proliferation rate, stronger

and invasive capacity compared with OA FLS [237,240]. Pathway analyses further indicate that although both OA and RA FLS involve TNF signaling, RA FLS are specifically enriched in chemokine signaling pathways, with key genes such as CCR5, CCR7, CXCR4, CCL5, and CCR4. In contrast, OA FLS show enrichment in genes such as SELE, SERPINE1, and NFKBIA, reflecting distinct regulatory and inflammatory programs [240]. At the subset level, genes associated with CD55⁺ lining FLS are more highly expressed in OA compared with leukocyte-rich RA. In contrast, HLA-DRA^{hi} (MHC class II-related) FLS and CD34⁺ sublining FLS are enriched in leukocyte-rich RA compared with OA, consistent with a stronger immune-interacting fibroblast phenotype in RA [182].

Unlike in OA, treatments for RA specifically target these immunological mechanisms. In DMARD (disease-modifying anti-rheumatic drug)-naïve patients with moderate to severe RA, initial therapy typically involves a conventional synthetic DMARD (csDMARD), with methotrexate (dihydrofolate reductase inhibitor) being the preferred option. In cases of mild disease, alternative csDMARDs such as sulfasalazine or hydroxychloroquine may be considered. Glucocorticoids are not recommended for long-term treatment. They may be used as bridging therapy when initiating or modifying a csDMARD but should be tapered and discontinued as early as clinically appropriate. In patients with inadequate response to csDMARDs and high disease activity, the addition of a biologic DMARD (bDMARD), such as a TNF inhibitor, or a targeted synthetic DMARD (tsDMARD), such as a JAK inhibitor, is advised. If methotrexate is contraindicated or not tolerated, an IL-6 receptor inhibitor may be used as monotherapy. In cases of persistent disease activity, switching to another TNF inhibitor or to another bDMARD, such as abatacept (a T-cell co-stimulation modulator) or rituximab (an anti-CD20 monoclonal antibody targeting B cells) is recommended [241,242].

To better highlight the common and distinct features of synovitis across physiological and pathological conditions, a comparative summary of normal synovium, OA, and RA is provided (Table 4) [175,207].

Feature	Normal synovium	OA synovitis	RA synovitis
Lining layer	Thin (1–2 cells), organized barrier	Mild to moderate hyperplasia	Marked hyperplasia, disorganization, pannus formation
Sublining cellularity	Low, sparse fibroblasts and immune cells	Increased, heterogeneous	Strongly increased, dense cellular infiltrate
Leukocyte infiltration	Minimal (resident macrophages, few lymphocytes)	Moderate, mainly macrophages and T cells, diffuse	High, dense and often organized (lymphoid aggregates), including B cells and plasma cells
Macrophages	Resident macrophages contributing to barrier function	Increased numbers, activated state	Markedly increased numbers, associated with inflammatory cytokine production
T cells	Rare	Present, heterogeneous subsets	Abundant, including Th1 and Th17 subsets
B cells / plasma cells	Absent or rare	Low levels	Prominent, with local antibody production
Mast cells	Rare	Increased, associated with synovitis severity	Increased
Angiogenesis	Basal vascular network	Increased vessel density	Strongly increased vascularization
Fibrosis	Absent	Present, mainly in later stages	Present
FLS phenotype	Homeostatic	Activation restricted to specific sublining subsets	Broad activation, proliferative and invasive phenotype
FLS organization	Clear lining vs sublining segregation	Partially altered	Strongly altered, loss of organization
Cytokine environment	Low	Moderate (e.g., IL-6, TNF- α , IL-1 β)	High (e.g., TNF- α , IL-1 β , IL-6, IL-17, IFN- γ)
Overall inflammation	None	Low to moderate, heterogeneous	High, persistent

Table 4. Comparative features of synovitis in normal synovium, OA and RA. OA: osteoarthritis; RA: rheumatoid arthritis.

3.5. Endoplasmic reticulum stress

The endoplasmic reticulum (ER) is an intracellular organelle responsible for proper protein folding, post-translational modifications, and trafficking, relying on chaperones such as heat shock proteins, calreticulin, and protein disulfide isomerases. Under pathological conditions (hypoxia, nutrient deprivation, or ER Ca^{2+} depletion), unfolded or misfolded proteins accumulate within the ER lumen, leading to ER stress. To restore homeostasis, cells activate the unfolded protein response (UPR), a conserved adaptive mechanism regulated by three ER-resident membrane sensors: inositol-requiring enzyme 1 α (IRE1), protein kinase RNA-like ER kinase (PERK), and activating transcription factor 6 (ATF6) (Figure 13) [11].

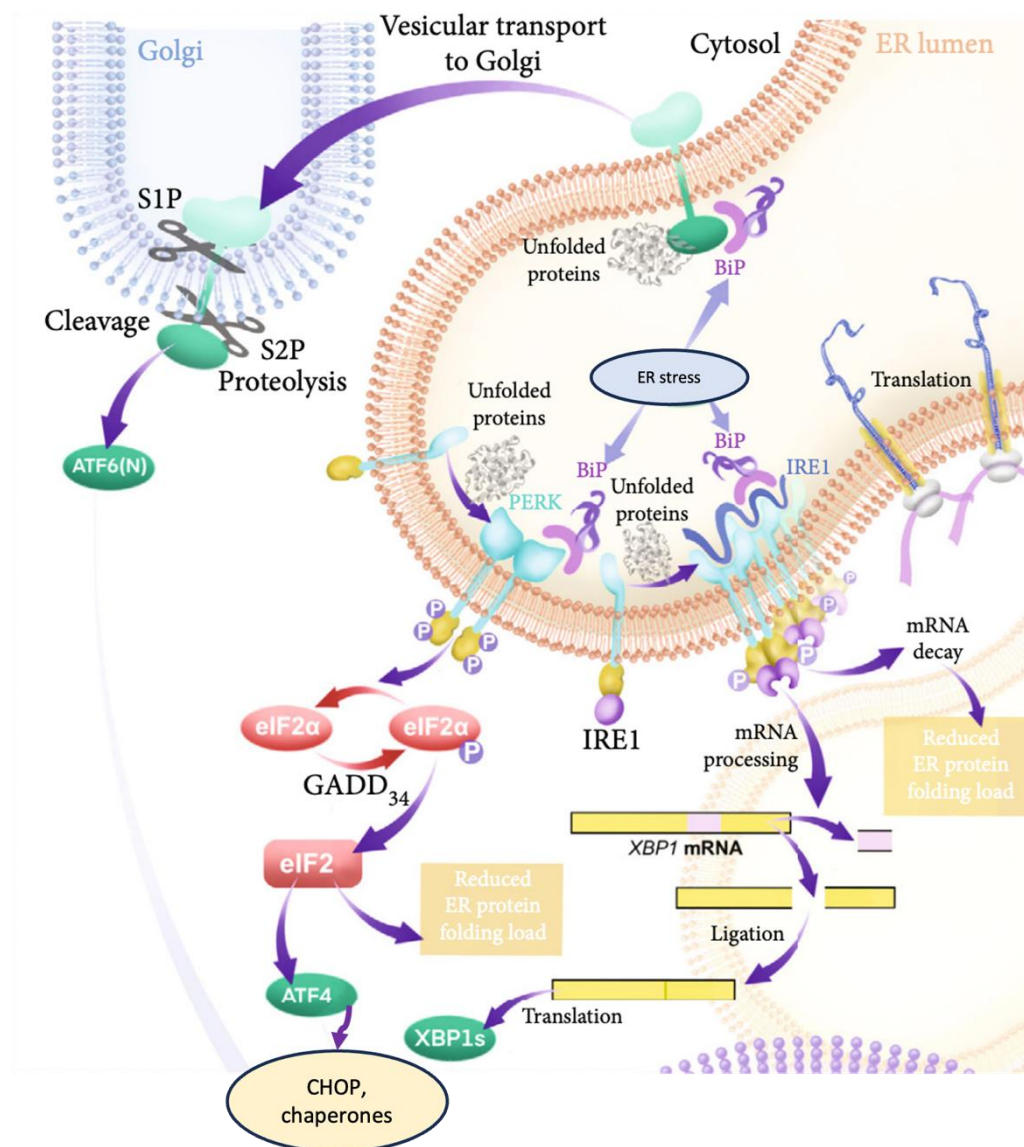


Figure 13. Under ER stress, the accumulation of unfolded proteins leads to the dissociation of BiP from three major ER stress sensors: IRE1 α , PERK, and ATF6, initiating the UPR. ATF6 translocates to the

Golgi where it is cleaved and functions as a transcription factor that induces chaperone and XBP1 expression. Activated PERK phosphorylates eIF2 α , reducing global translation and promoting ATF4 production, which regulates stress, chaperones and apoptosis response genes. IRE1 splices XBP1 mRNA, producing the active transcription factor XBP1s, which enhances protein folding and degradation capacity. Together, these pathways aim to restore ER homeostasis by reducing the protein load and increasing the folding and degradation machinery. ATF4: Activating Transcription Factor 4; ATF6: Activating Transcription Factor 6; eIF2 α : eucaryotic Initiation Factor 2 α ; IRE1 α : Inositol Requiring Enzyme 1 α ; PERK: PKR-like Endoplasmic Reticulum Kinase; XBP-1: X-box Binding Protein 1; BiP: Binding immunoglobulin protein; GADD34: growth arrest and DNA damage-inducible protein; CHOP: C/EBP Homologous Protein. Adapted from [243].

Binding immunoglobulin protein (BiP), also known as glucose-regulated protein 78 (GRP78) or heat shock protein 5 (HSPA5), plays a central role in the ER stress regulation. Under basal conditions, BiP is bound to the three major ER stress sensors: PERK, IRE1 α and ATF6. When misfolded proteins accumulate, BiP dissociates from these sensors to bind aberrant proteins, thereby allowing activation of the UPR and the degradation of proteins through the ER-associated degradation (ERAD) system, which targets misfolded proteins for ubiquitination and proteasomal degradation.

The PERK branch is usually kept inactive by binding to BiP [244,245]. Upon stress, BiP dissociates from PERK, allowing its autophosphorylation and activation. PERK then phosphorylates the translation initiation factor eIF2 α , leading to a general reduction in protein synthesis, thereby decreasing the burden of nascent polypeptides entering the ER. However, some transcripts, such as that of ATF4, are selectively translated. ATF4 promotes the expression of chaperones and genes involved in antioxidant responses, but also the pro-apoptotic transcription factor C/EBP Homologous Protein (CHOP) when stress is prolonged or unresolved [11,243].

Similarly, IRE1 α is activated upon BiP release, followed by oligomerization and autophosphorylation. Its endoribonuclease domain becomes active, enabling the unconventional splicing of X-box binding protein 1 (XBP1) mRNA into sXBP1, which supports chaperones and ERAD gene expression. Additionally, IRE1 α mediates regulated “IRE1-dependent decay” (RIDD), degrading various mRNAs to reduce the influx of proteins into the ER [246,247].

Upon ER stress, ATF6 translocates to the Golgi apparatus, where it is cleaved by site-1 and site-2 proteases. The resulting N-terminal fragment functions as a transcription factor in the nucleus, promoting the expression of ER chaperones and of XBP1. In its active spliced form

(sXBP1), XBP1 induces the transcription of additional ER chaperones and ERAD components, which target misfolded proteins for ubiquitination and proteasomal degradation [11,243,248].

In summary, under mild or transient ER stress, the UPR promotes adaptive mechanisms such as chaperone upregulation, reduced translation, and degradation of misfolded proteins. However, if the stress is severe or prolonged, these protective responses are overwhelmed, leading to inflammation [249,250].

3.6. ER stress & inflammation

Links between ER stress and inflammation are represented in Figure 14. Under prolonged cellular disturbance, nuclear factor-kappa B (NF- κ B) is released from its cytoplasmic inhibitor, I kappa B (I κ B), which is first phosphorylated and subsequently degraded. NF- κ B plays a pivotal role in linking ER stress to the inflammatory response, as its nuclear translocation leads to the transcription of multiple proinflammatory genes and cytokines, including IL-6 [11,250]. This process is mediated by the three UPR sensors, which acquire proinflammatory properties under sustained ER stress. Once activated, IRE1 α recruits TNF receptor-associated factor 2 (TRAF2), leading to the activation of I κ B kinase (IKK) [251,252]. IKK phosphorylates I κ B, enhancing its degradation and enabling NF- κ B to enter the nucleus and induce inflammatory gene transcription [11]. Interestingly, mild ER stress can inhibit this pathway by reducing TRAF2 expression, thereby limiting I κ B degradation and favoring apoptosis instead [251]. In parallel, the IRE1 α –TRAF2 complex activates c-Jun N-terminal kinase (JNK), which in turn phosphorylates and activates the transcription factor AP-1, further promoting proinflammatory gene expression [11,253,254]. This axis is also influenced upstream by NOD1/2-RIPK2 signaling [253]. Additionally, spliced XBP1 contributes to inflammation by inducing the production of cytokines such as IL-6, IL-8, and IFN- γ [255].

The PERK–eIF2 α pathway amplifies NF- κ B activity by reducing I κ B mRNA translation [256,257]. PERK also promotes translation of ATF4, which enhances IL-6 and IL-8 secretion in endothelial cells [255]. ATF6, once activated, stimulates the PI3K/AKT signaling cascade and directly induces transcription of inflammatory genes [258]. Furthermore, cleaved ATF6 fragments are implicated in the acute phase response, promoting the release of proinflammatory cytokines (IL-1, TNF- α , IL-6) and acute phase proteins like CRP [259]. ATF6 also facilitates NF- κ B activation [255].

Both PERK and IRE1 α can activate the PI3K/AKT pathway, further linking ER stress to inflammatory signaling [11]. Reactive oxygen species (ROS), generated during UPR through protein disulfide isomerase (PDI)-mediated redox reactions, also contribute to inflammation by activating JNK and downstream AP-1 [255,260]. Although the PERK pathway can induce the antioxidant factor Nrf2 to counteract oxidative stress, prolonged ER stress ultimately leads to ROS accumulation [261].

Finally, the metaflammasome—a multiprotein complex responsive to ER stress, nutrients, and pathogens—also integrates into this network. Its core component, the eIF2 α kinase PKR, interacts with key signaling molecules such as IKK, JNK, IRS1, and eIF2 α itself, further amplifying the inflammatory response [255,262].

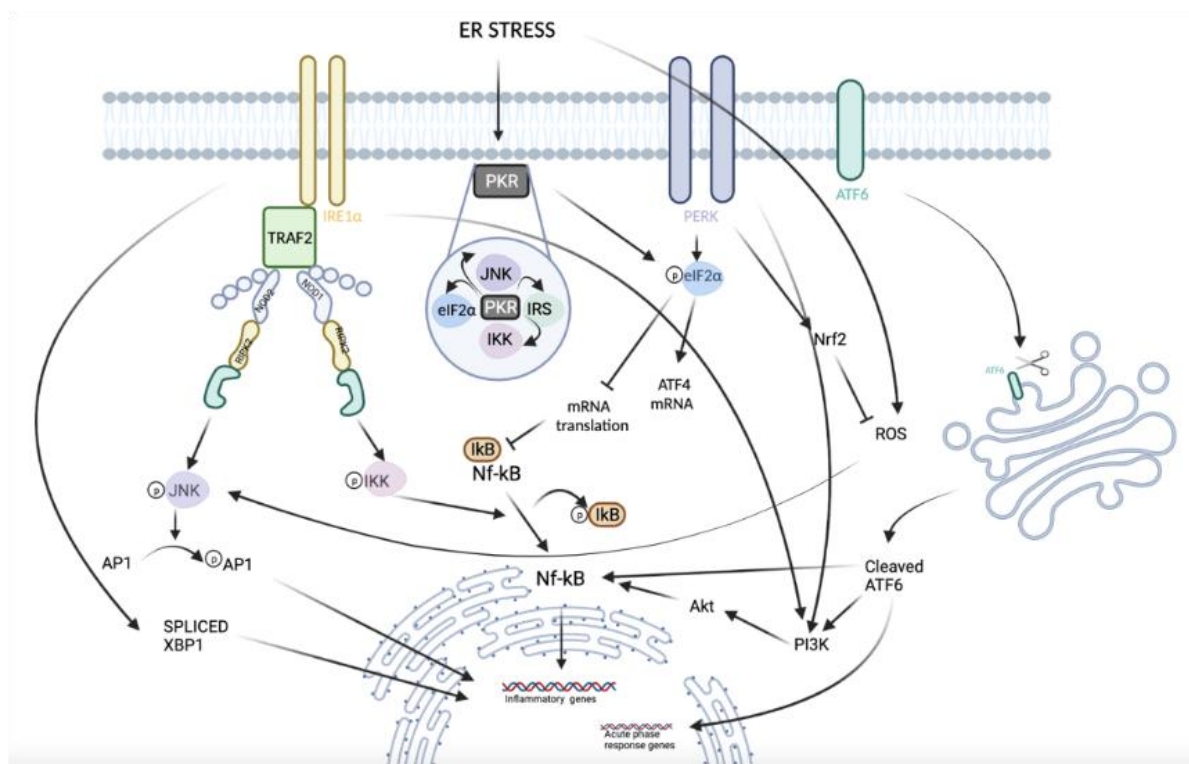


Figure 14. Integration of ER stress and inflammation through UPR signaling pathways. The three main UPR sensors—IRE1 α , PERK, and ATF6—are activated upon BiP dissociation in response to misfolded protein accumulation. IRE1 α recruits TRAF2 to activate JNK and IKK, promoting NF- κ B and AP-1-mediated transcription of inflammatory genes. Spliced XBP1 and the NOD1/2–RIPK2 pathway also contribute to cytokine production. PERK phosphorylates eIF2 α , leading to ATF4 translation, reduced I κ B synthesis, and increased NF- κ B activity, while also modulating redox balance through ROS and Nrf2. Cleaved ATF6 activates inflammatory signaling via the PI3K/Akt pathway and drives the acute phase response. PKR, a component of the metaflammasome, links ER stress to inflammatory kinases like IKK and JNK. Together, these pathways connect sustained ER stress to proinflammatory responses. IRE1 α , Inositol-Requiring Enzyme 1 α ; PERK, PKR-like ER kinase; ATF6, Activating Transcription Factor 6; TRAF2, TNF Receptor-Associated Factor 2; JNK, c-Jun N-terminal Kinase; IKK, I κ B Kinase; NF- κ B, Nuclear Factor kappa-light-chain-enhancer of activated B

cells; AP-1, Activator Protein 1; XBP1, X-box Binding Protein 1; NOD1/2, Nucleotide-binding Oligomerization Domain 1/2; RIPK2, Receptor-Interacting Protein Kinase 2; eIF2 α , Eukaryotic Initiation Factor 2 alpha; ATF4, Activating Transcription Factor 4; I κ B, Inhibitor of NF- κ B; ROS, Reactive Oxygen Species; Nrf2, Nuclear Factor Erythroid 2–Related Factor 2; PI3K, Phosphatidylinositol 3-Kinase; PKR, Protein Kinase R; CRP, C-Reactive Protein. Adapted from [11] made with bioRender.

3.7. ER stress & synovitis

A link has been demonstrated between ER stress and synovitis, creating a self-perpetuating circle.

Synovitis is characterized by sustained activation of inflammatory pathways, leading to increased production of cytokines by resident and infiltrating immune cells, together with expansion and activation of FLS. This inflamed microenvironment, often associated with oxidative stress, hypoxia, limited nutrient availability, and acidic conditions, places a substantial burden on the ER by increasing protein-folding demand and perturbing ER homeostasis, ultimately triggering activation of the UPR (Figure 15) [11].

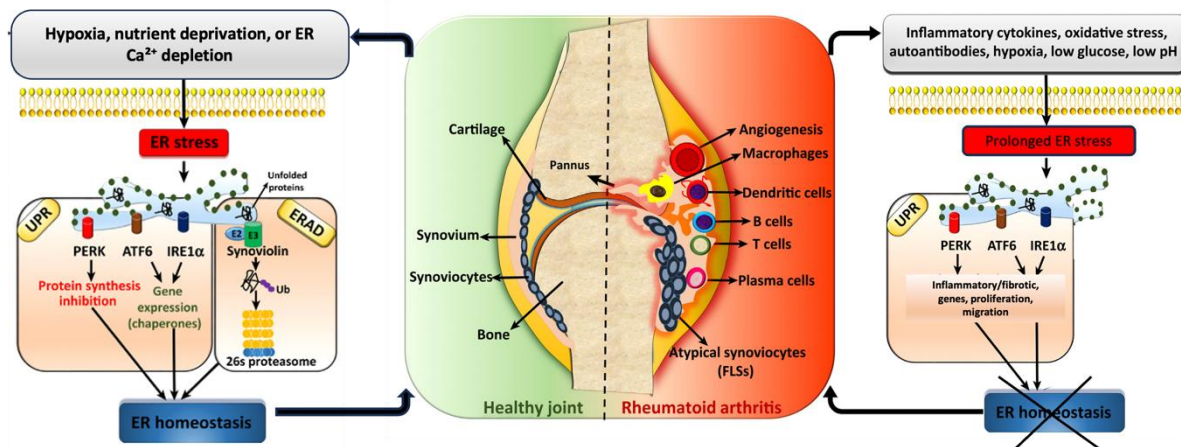


Figure 15. Model linking ER stress to synovitis. In healthy joints, transient stressors such as hypoxia, nutrient deprivation, or Ca²⁺ imbalance can induce ER stress, which is resolved through adaptive UPR signaling (PERK, ATF6, and IRE1 α) to restore ER homeostasis via translational control, chaperone induction, and ERAD. During synovitis, FLS and infiltrating immune cells together shape an inflammatory microenvironment enriched in cytokines, oxidative stress, and metabolic constraints (hypoxia, low glucose, low pH). These sustained cues promote prolonged ER stress and persistent UPR activation in synovial cells, driving inflammatory and fibrotic gene programs as well as FLS proliferation and migration, thereby contributing to synovial pathology. ER: endoplasmic reticulum; UPR: unfolded protein response; FLS: fibroblast-like synoviocytes; IRE1 α , Inositol-Requiring Enzyme 1 alpha; PERK, PKR-like ER kinase; ATF6, Activating Transcription Factor 6; ERAD: ER-associated degradation; adapted from [11].

Endoplasmic reticulum stress components, including BiP, eIF2 α , the active/cleaved forms of ATF6, IRE1, XBP-1, and elements of the ubiquitin–proteasome pathway, are overexpressed in FLS from RA patients [263,264]. BiP is highly expressed in both the lining and sublining layers of RA synovial tissue [11,265]. It contributes to FLS proliferation and promotes angiogenesis in RA [263]. In line with this, a recent study showed that IL-17 drives similar effects in OA FLS and is associated with BiP overexpression [266]. The PERK/eIF2 α branch also activates NF- κ B signaling in MLS and FLS, contributing to the development of arthritis. Its downstream effector, GADD34, is overexpressed in peripheral blood mononuclear cells (PBMCs) from RA patients [11]. PERK and ATF6 are both expressed in inflammatory and fibrotic OA FLS populations, and their expression, as well as the resulting synovitis, can be attenuated by the flavonoid chrysin [267]. Inhibition of IRE1 α reduces TLR-induced inflammatory cytokine production and thereby alleviates arthritis [268]. Activation of the IRE1 α pathway induces autophagy and promotes FLS activation, proliferation, and migration in RA [265]. TLR-mediated activation of XBP-1 in FLS promotes the secretion of pro-inflammatory cytokines such as IL-6 and TNF- α [269]. ER-resident E3 ubiquitin ligase synoviolin is associated with FLS proliferation and protects cells from ER stress-induced apoptosis [270,271].

Independently of the three UPR branches becoming skewed and secondarily promoting NF- κ B activation, chaperone proteins may themselves exert significant pro-inflammatory effects when secreted or displayed on the cell surface [272]. Interestingly, several studies have reported the presence of autoantibodies directed against ER chaperones such as calreticulin, BiP, HSP90B1, and PDI family in various autoimmune diseases [272]. Specifically, extracellular CALR can directly activate the NF- κ B pathway in macrophages by promoting I κ B α degradation, thereby enhancing the transcription of TNF- α and IL-6, as shown in patients with RA and systemic lupus erythematosus [273]. CALR acts as a ligand for LRP1 on macrophages in experimental phagocytic models and triggers the phagocytosis of apoptotic cells [274]. Particularly, extracellular chaperones also play a role in RA. CALR is overexpressed in synovial fluid of RA patients, reflecting its extracellular expression in this context [275]. BiP, HSP90B1 and CALR can function as autoantigens, leading to autoantibody production and amplification of the immune response in RA [276–278]. Within the synovial tissue, BiP has also been reported at the surface of synoviocytes, with a possible increase in RA FLS compared with OA FLS; however, this observation was obtained from a single OA and a single RA sample and should therefore be interpreted with caution [277]. However, the precise contribution of extracellular chaperone proteins to the initiation of synovitis remains poorly understood.

3.8. Previous work

Our laboratory previously identified, by mass spectrometry, the bulk of ER stress proteins whose expression correlated with the Tak score in synovial membranes from patients with OA, CPPD and RA [13]. Synovial biopsies were analyzed using high-resolution liquid chromatography–tandem mass spectrometry, allowing the identification of 4,336 proteins, of which 1,871 were retained based on robust detection criteria. Protein abundance was quantified using label-free quantification and correlated with the Tak score reflecting synovial inflammation, thereby revealing a continuous inflammatory gradient across OA, CPPD and RA. Among these, 51 proteins showed a strong and significant correlation with the histological score ($p < 0.001$), from which a subset of ER-resident proteins was identified (Figure 16).

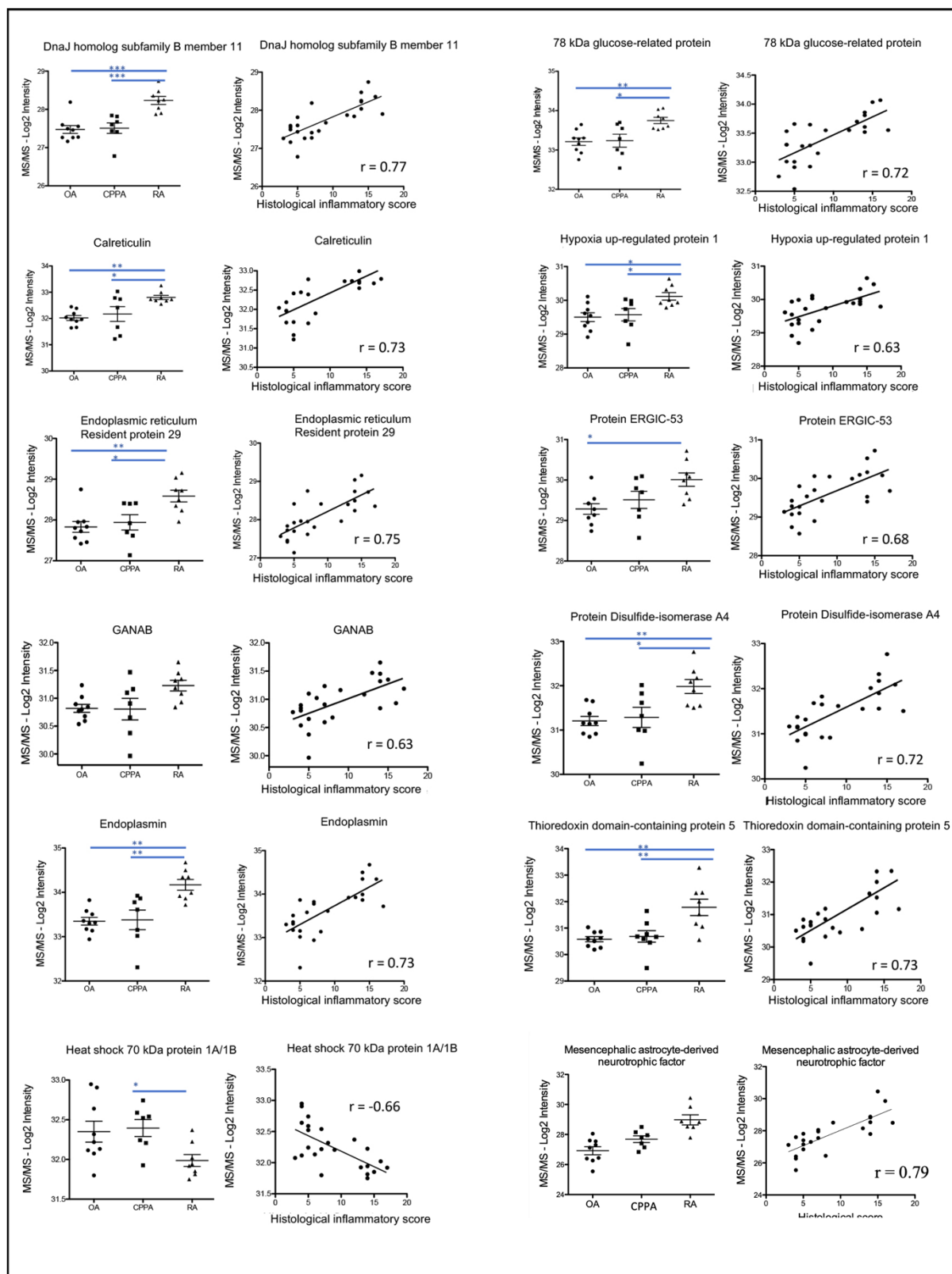
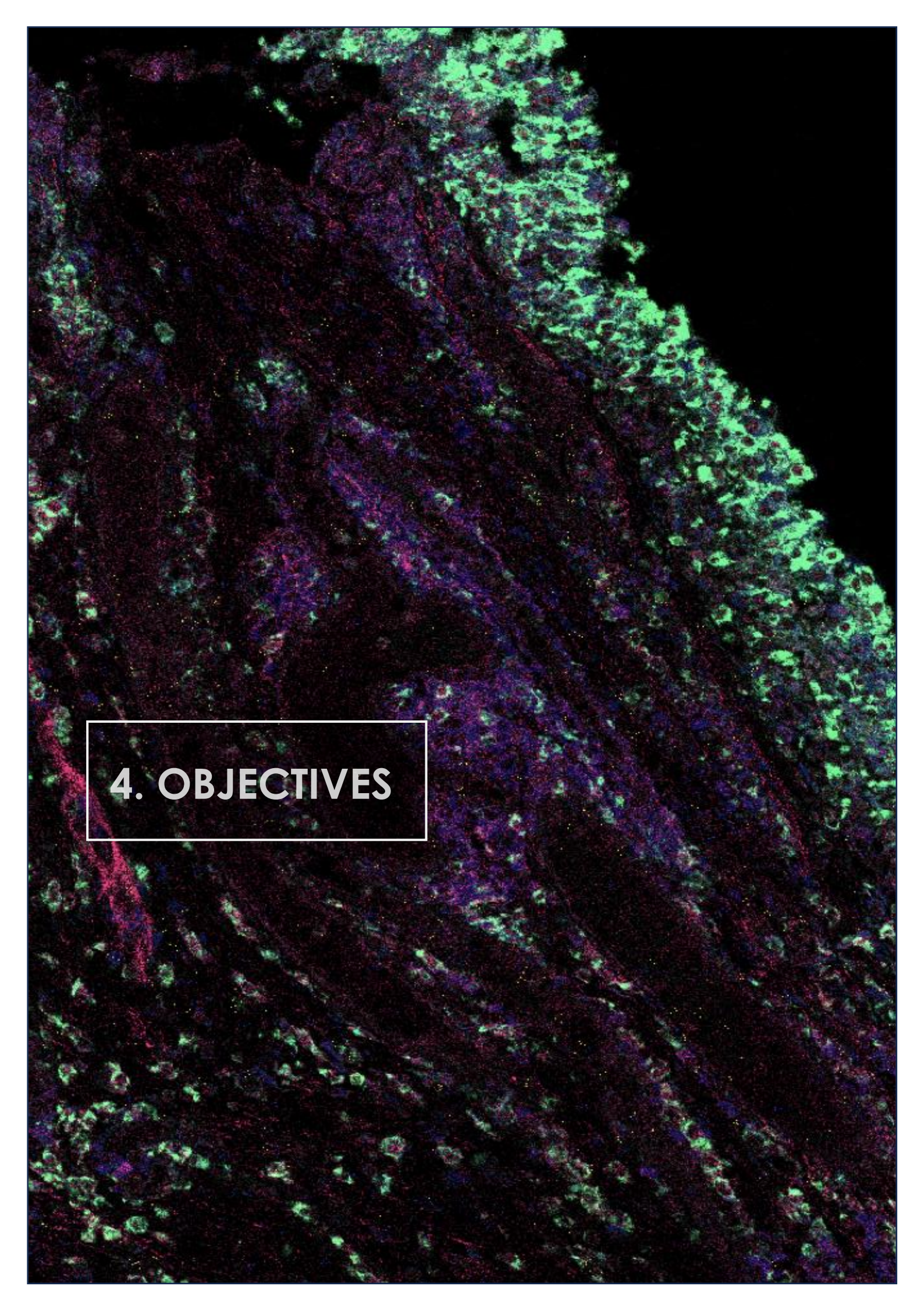


Figure 16. Graphs from de Seny *et al.* [13], showing the distribution of ER protein abundances across OA, CPPD, and RA groups and correlation with histological inflammatory Tak score. Log2-transformed intensities of the 11 ER proteins identified by LC–MS/MS are shown across the three groups (OA, CPPD, and RA) and their association with the histological inflammatory score is indicated. *, **, *** indicate P-values < 0.05, < 0.01, and < 0.001, respectively. OA : osteoarthritis ; CPPA/CPPD : chronic pyrophosphate arthropathy/disease; RA: rheumatoid arthritis.

Among these, eleven ER-resident proteins were significantly associated with increasing inflammatory scores, including molecular chaperones involved in protein folding (BiP/GRP78, HSP90B1/Endoplasmin, DNAJB11), oxidoreductases (PDIA4, TXNDC5), glycoprotein processing proteins (LMAN1/ERGIC-53, GANAB), and ER stress-adaptive factors (HYOU1, MANF, ERP29 and CALR). The progressive increase in their abundance along the OA–CPPD–RA continuum (Figure 16) highlights the activation of ER proteostasis pathways in proportion to synovial inflammation.



4. OBJECTIVES

Based on the identification of a broad panel of ER stress proteins whose expression correlated with the TAK inflammatory score across OA, CPPD, and RA synovial membranes, and given the continuous spectrum of inflammation observed in these biopsies, we sought to better understand the biological significance of this association. The key question that emerged was whether the upregulation of these ER stress chaperones merely reflects a secondary adaptive response to inflammatory stress, or whether these proteins actively contribute to the initiation and progression of synovitis.

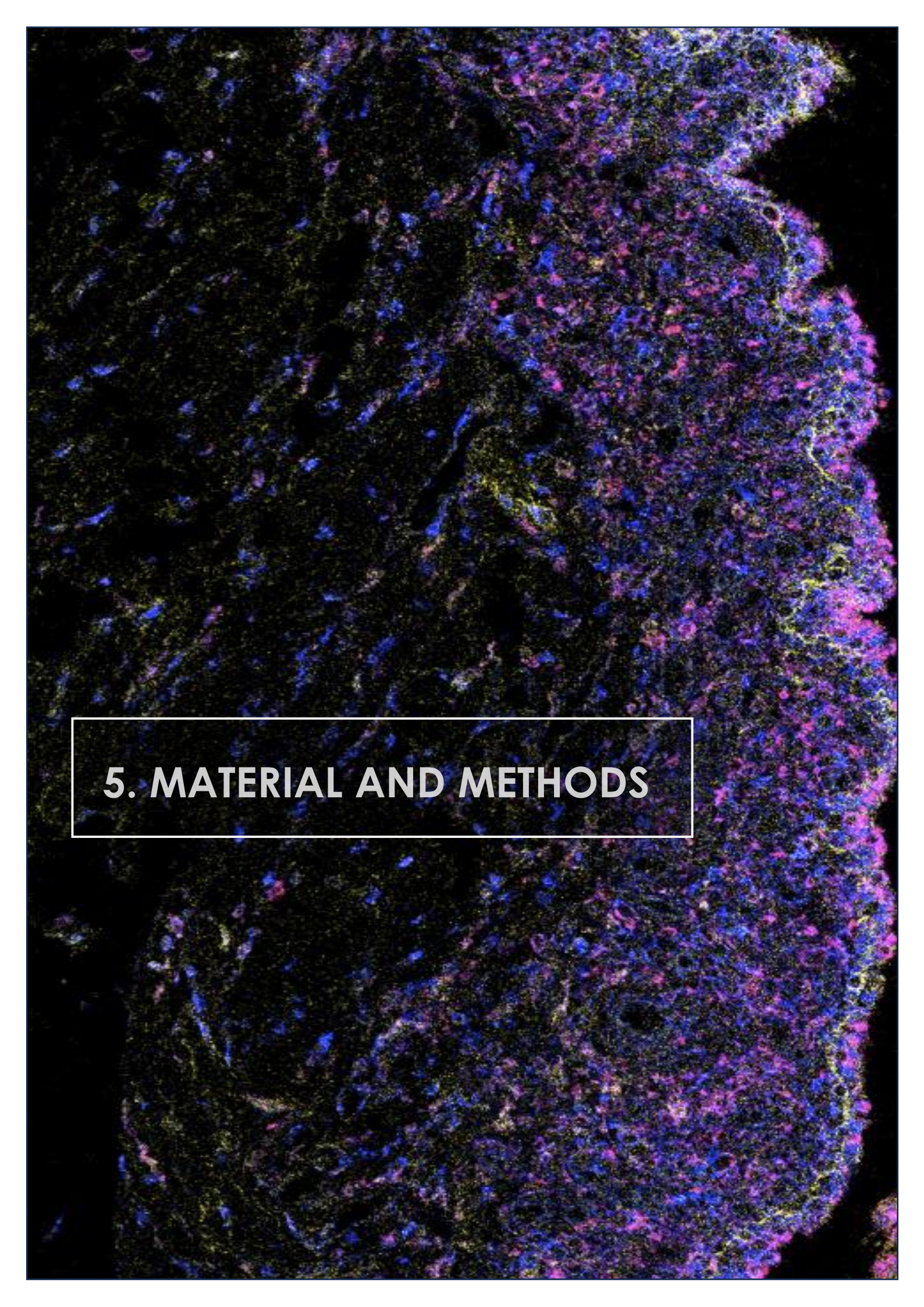
As molecular chaperones, many of these proteins are classically involved in maintaining cellular homeostasis by assisting protein folding and restoring ER balance under stress conditions. Their increased expression could therefore represent a protective, compensatory response aimed at limiting inflammation-induced cellular dysfunction. Alternatively, these chaperones may play an active pathogenic role in synovitis initiation, potentially contributing to key features of the inflamed synovium such as FLS proliferation (hyperplasia), leukocyte recruitment and inflammatory amplification, pro-fibrotic remodeling, or even extracellular signaling if secreted upon ER stress induction.

To address these hypotheses and determine whether these proteins act as adaptive regulators or as drivers of synovial pathology, we will apply this following experimental strategy:

1. We will first confirm by IHC that the bulk of ER stress proteins identified by mass spectrometry is indeed expressed in the same formalin-fixed, paraffin-embedded (FFPE) synovial membranes than those used for the proteomic approach.
2. We will next validate these observations on the Collagenase-Induced Synovitis (CIS) Mouse Model, which will overcome the limitations associated with human samples and allow for a comparison of protein expression level between healthy controls and OA models.
3. We will next investigate by imaging mass cytometry (IMC) which key cell subpopulations within the inflamed synovial membranes express these proteins.
4. Based on the specific cell subpopulation expressing these proteins, we will assess *in vitro* how their expression is modulated within these cells after induction of an ER stress, inflammatory stimuli, or fibrotic processes, and determine whether these proteins are secreted upon ER stress induction. In parallel, we will determine through which UPR

branches—IRE1 α , ATF6 or PERK—their regulation occurs, in order to better define the signaling pathways involved.

5. We will then select the proteins most strongly modulated under these stimulation conditions in order to investigate their functional role in promoting pro-fibrotic, pro-inflammatory and/or proliferative responses within the same cell subpopulations.



5. MATERIAL AND METHODS

5.1. Human synovial biopsies

All human sample analyses were conducted in accordance with the ethical regulations of the University Hospital of Liège (CHU, ref: 2017/147) and the Catholic University of Louvain (UCLouvain, ref:2013/24MAI/293), and received approval from their respective ethical committees as well as from the biobank research committees (CHU - BB190058, UCLouvain –BB190044).

Synovial biopsies were obtained from clinically affected knee joints using needle arthroscopy. Three to five synovial fragments were collected per patient. Samples were either embedded in paraffin for immunohistochemical analyses or stored at –80 °C until use for proteomic studies. Three independent cohorts were established based on their use for specific experimentations. Demographic, biologic, and inflammatory data are listed in Table 5.

	Cohort 1 CHU Liège			P value	Cohort 2 UCLouvain		Cohort 3 CHU Liège
	OA	CPA	RA		OA	RA	
n	9	7	8		2	1	OA
Gender (W/M)	8/1	5/2	5/3	0.44	1/1	1/0	2.5
Age [mean (+/- SD)] year	57.4 (+/-14.1)	64.6 (+/-8.5)	55.75 (+/-16.3)	0.3	78 (+/-5.6)	58	21/4
BMI [mean (+/- SD)] kg/m2	30.3 (+/-7.3)	26.5 (+/-4.3)	25.2 (+/-6)	0.4	28.7 (+/-6.7)	23.83	65 (+/-14.3)
Anti-ACPA			4/8			0	28.9 (+/- 5.3)
Rheumatoid factor			3/8			0	
Tak score [mean (+/- SD)]	4.2 (+/-1.8)	5.7 (+/-2.1)	12.2 (+/-1.5)	0.0003	2 (+/-2.8)	13	4.3 (+/-2)
Kellgren-Lawrence grade	2.7 (+/-1.6)						2.7 (+/-1.2)
Immunohistochemistry		yes				no	no
Imaging mass cytometry		no				yes	no
Immunofluorescence		no				no	yes

Table 5. Demographic, biologic (ACPA, RF), Tak score data, Kellgren-Lawrence grade and specific use for each cohort. n: number of patients; W: women; M: men; SD: standard deviation; BMI: body mass index; ACPAs: anti-citrullinated protein antibody; RF: rheumatoid factor.

Samples recruitment for each cohort and their allocation to specific experimental techniques is described below (Figure 17).

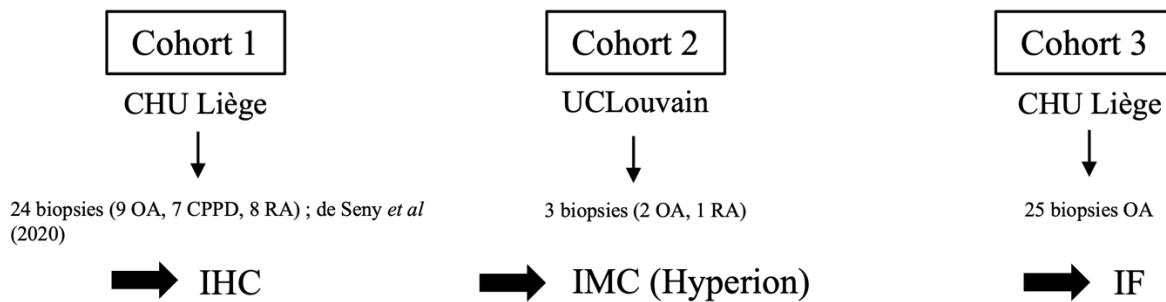


Figure 17. Flowchart of cohort definition. OA: osteoarthritis; CPPD: chronic pyrophosphate disease; RA: rheumatoid arthritis; IHC: immunohistochemistry; IMC: imaging mass cytometry; IF: immunofluorescence.

- **Cohort 1** (n = 24): samples from CHU de Liège, consisting of 9 OA, 7 CPPD and 8 RA. This cohort was used for IHC. OA and CPPD synovial biopsies were performed at variable time points after disease onset, ranging from early to more established stages. For RA, synovial biopsies were performed at the time of diagnosis in treatment-naïve patients, with no prior exposure to DMARDs or biologic therapies.
- **Cohort 2** (n=3): FFPE initially collected from 50 biopsies at UCL. Among them, 2 OA and 1 RA. These were selected as representing the two main disease groups of the study; they were processed by IMC. For this cohort, synovial biopsies were performed at defined time points after disease onset. The RA sample was collected 4 years after disease onset, while the two OA samples were obtained 1 year (Tak 0) and 20 years (Tak 4) after disease onset.
- **Cohort 3** (n = 25): additional CHU Liège samples from 25 OA patients and used for IF. The synovial biopsies were performed at variable time points after disease onset, ranging from early to more established stages.

For each cohort, the selected synovial biopsies collectively spanned a continuous gradient of inflammation, as assessed by the modified Tak score. This semiquantitative score, hereafter referred to as the “Tak score” (Table 6), evaluates lining layer hyperplasia and inflammatory cell infiltration, including neutrophils, lymphocytes, and plasma cells, while excluding vascular parameters [211]. Histological scoring was performed on one section per patient in a blinded

manner by Dr. E. Bianchi (anatomopathology), without access to clinical data or underlying diagnosis.

Cohort	Pathology	Histological scores				Tak score
		Hs	Ly	Pl	Po	
1	OA	1	1	0	0	2
		2	1	0	0	3
		2	1	0	0	3
		2	1	0	0	3
		2	1	0	0	3
		3	2	0	0	5
		3	1	2	0	6
		3	2	1	0	6
		4	2	1	0	7
	CPPA	3	1	0	0	4
		3	1	0	0	4
		2	1	2	0	5
		3	2	0	0	5
		4	2	0	0	6
		2	3	1	0	6
	RA	4	3	1	2	10
		2	4	2	3	11
		4	3	1	2	11
		4	3	3	1	11
		4	3	4	0	11
		4	4	4	0	12
		3	4	4	3	14
		4	4	4	2	14
		4	4	4	2	14
2	OA	0	0	0	0	0
		1	2	1	0	4
	RA	2	4	4	3	13
3	OA	1	1	0	0	2
		0	2	0	0	2
		0	2	0	0	2
		1	1	0	0	2
		2	1	0	0	3
		2	1	0	0	3
		2	1	0	0	3
		3	1	0	0	3
		2	1	0	0	3
		3	1	0	0	4
		2	1	1	0	4
		2	1	1	0	4
		2	2	1	0	5
		2	2	1	0	5
		2	1	2	0	5
		3	2	0	0	5
		2	3	0	0	5
		3	2	0	0	5
		3	2	1	0	6
		3	1	2	0	6
		3	2	1	0	6
		4	2	1	0	7
		4	3	1	0	8
		4	3	3	0	10

Table 6. Detailed Tak score for each cohort. The score was modified by excluding vascular parameters (hereafter referred to as “Tak score”) OA: osteoarthritis; CPPD: chronic pyrophosphate disease; RA: rheumatoid arthritis; Hs: hyperplasy; Ly: lymphocytes; Pl: plasmocytes; Po: Polymorphonuclear leukocytes.

5.2. Collagenase induced synovitis mouse model (CIS)

Animal experiments were conducted in accordance with institutional guidelines and approved by the local ethical committee (University of Liège, Belgium). The CIS mouse model was used as previously described by Deroyer *et al.* [200]. Eight-week-old male C57BL/6 mice were anesthetized with isoflurane, followed by two I-A injections of collagenase VII (1 U per knee, Sigma-Aldrich, St. Louis, USA), administered 48 hours apart. Control animals received injections of saline. Mice were sacrificed one week after the second injection, and knee joints were collected. Successful induction of synovitis was confirmed by increased expression of IL1 β and IL6 mRNA in the synovial membrane (assessed by RT-qPCR), along with increased synovial thickness. Early fibrotic changes were further supported by Acta2 mRNA overexpression [200]; fibrosis was not directly assessed using dedicated histological scoring methods [214]

5.3. Immunohistochemistry

IHC analyses were performed on both human and murine synovial membranes. Human synovial tissues from Cohort 1 (ULiège) were fixed in 4% paraformaldehyde for 24 hours, dehydrated in 70% ethanol, and subsequently embedded in paraffin. For murine samples, knee joints were fixed in the same manner and decalcified in EDTA for 15 days prior to dehydration and paraffin embedding. Sections underwent heat-mediated antigen retrieval in a steamer using Target Retrieval Solution (Agilent), followed by blocking of endogenous peroxidase activity and non-specific binding sites using hydrogen peroxide and Dako Real antibody diluent (Agilent), respectively.

Human sections were incubated with primary antibodies targeting selected ER chaperone proteins (Table 6). For murine tissues, only a subset of antibodies (BiP, MANF, PDIA4, HSP90B1, CALR, DNAJB11, and LMAN1) was retained based on cross-species compatibility. The corresponding gene names are listed in Table 7.

		IHC	CIS
Gene ID	Protein name	Reference	Compatibility
<i>BiP</i>	Binding immunoglobulin protein (BiP)	#3177, Cell signaling	X
<i>HYOU1</i>	Hypoxia up-regulated protein 1 (GRP170)	#ab134944	
<i>MANF</i>	Mesencephalic astrocyte-derived neurotrophic factor	#ab67271	X
<i>PDIA4</i>	Protein disulfide-isomerase A4	#ab155800	X
<i>GANAB</i>	α -subunit of glucosidase II	#ab176349	
<i>HSP90B1</i>	Heat shock protein 90 beta family member 1	#20292, Cell signaling	X
<i>TXNDC5</i>	Thioredoxin domain-containing protein 5	#CF507281, Origen	
<i>DNAJB11</i>	DnaJ homolog subfamily B member 11	#ab224082	X
<i>LMAN1</i>	Lectin mannose- binding 1	#ab125006	X
<i>ERP29</i>	Endoplasmic reticulum resident protein 29	#ab176573	
<i>CALR</i>	Calreticulin	#ab92516	X

Table 7. List of gene and protein references corresponding to the 11 ER chaperone proteins analyzed by IHC (with respective antibody reference) and/or by the CIS model. ER: endoplasmic reticulum; IHC: immunohistochemistry; CIS: Collagenase-Induced Synovitis.

Detection was performed using the EnVision+ HRP polymer system (Agilent) and DAB chromogen substrate (Agilent). Proteins were detected and semi-quantified using QuPath software, with normalization to total cell count [279].

5.4. Imaging mass cytometry

Imaging Mass Cytometry was performed using the Hyperion Imaging System (Standard BioTools™, California) on 3 selected FFPE synovial biopsies from the UCL cohort (Cohort 2), as described in the section "Human synovial biopsies". After deparaffinization and antigen retrieval, tissues were incubated with a panel of metal-conjugated antibodies targeting ER stress proteins and cell-type markers (Table 8). The cell-type panel was established based on recent single-cell studies characterizing synovial cell populations [182,204,205]. A DNA intercalator was included to enable nuclear staining and cell segmentation.

	IMC			
	Isotope	Clone	Reference	Cell/protein type
BiP	160Gd	EPR4041(2)	ab236050	ER stress protein
HYOU1	148Nd	201	MA5-29334	ER stress protein
MANF	172Yb	Rabbit Polyclonal	ab67271	ER stress protein
PDIA4	168Er	EPR14824(B)	ab251013	ER stress protein
GANAB	169Tm	EPR12376	ab249921	ER stress protein
HSP90B1	146Nd	EPR22847-50	ab256312	ER stress protein
TXNDC5	154Sm	OTI2E6	CF507281	ER stress protein
DNAJB11	145Nd	Rabbit Polyclonal	PA5-117915	ER stress protein
LMAN1	175Lu	EPR6979	ab248096	ER stress protein
ERP29	150Nd	EPR12499(B)	ab249928	ER stress protein
CALR	143Nd	EPR3924	ab271865	ER stress protein
CD34	139La	EP373Y	ab198395	Sublining fibroblast
CD55	149Nd	EPR6689	ab230797	Lining fibroblasts
CD31	151Eu	EPR3094	3151025D	Endothelial cells
CD4	156Gd	EPR6855	3156033D	Thelper
CD68	159TB	KP1	3159035D	Macrophages
CD20	161Dy	H1	3161029D	B cells
CD3	170Er	Polyclonal, C-Termin	3170019D	T cells

Table 8. List of ER stress proteins and cell-specific markers used for IMC, along with corresponding metal tags and antibody references. ER: endoplasmic reticulum; IMC: imaging mass cytometry

Each antibody was coupled to a unique metal isotope, allowing multiplexed spatial profiling of proteins by CyTOF-based imaging. Tissue regions of interest were ablated by laser, and ionized metal tags were detected and quantified according to their mass-to-charge ratio (m/z), yielding both quantitative and spatial data on the expression of each marker. Raw IMC data files (.mcd format) were converted into the standardized “.ome.tiff” format using a custom in-house Python script, preserving spatial metadata for compatibility with downstream pipelines. Image processing was conducted in QuPath (v0.5.1). Tissue classification was carried out using a pixel classifier trained via Random Forest to distinguish tissue from background based on DAPI signal. Nuclei segmentation was achieved using StarDist, integrated in QuPath, with a model specifically trained for histological nuclear features. Using the derived single-cell masks, the signal intensity for 18 markers (Table 8) was quantified per cell. Marker positivity thresholds were defined from the OA samples using the mean + 2 standard deviations, after excluding outliers beyond 1.5 times the interquartile range. Spatial clustering and downstream analyses

were conducted using CytoMAP (v1.4.21), employing a Self-Organizing Map (SOM) algorithm with nearest-neighbor context. The optimal number of clusters was defined using the Davies–Bouldin index.

5.5. Immunofluorescence on Formalin-fixed paraffin-embedded tissues

FFPE sections from Cohort 3 (ULiège – 25 OA samples) were incubated overnight at 65 °C. Following deparaffinization, antigen retrieval was performed using heat-induced epitope retrieval (HIER) in Target Retrieval Solution (Agilent). Endogenous peroxidase activity was blocked with H₂O₂ for 20 minutes. To reduce background signal and begin permeabilization, slides were incubated in a solution containing 0.4% Triton X-100 (CAS-9036195; Sigma Aldrich) and 125 mM glycine. After placement in a humidified chamber, non-specific binding sites were blocked using Protein Block Serum-Free (Agilent, X0909). Immunostaining was carried out in three sequential cycles, each including primary (Table 9) and corresponding secondary antibody incubations, separated by additional permeabilization and blocking steps:

- Step 1: mouse anti-CD34 and rabbit anti-PDIA4, followed by anti-mouse Alexa Fluor 594 (ab150116, Abcam) and anti-rabbit Alexa Fluor 532 (A11009, Thermo Fisher);
- Step 2: rabbit anti-CD55, followed by anti-rabbit Alexa Fluor 488 (ab150081);
- Step 3: directly conjugated antibodies anti-CD68-Alexa Fluor 647 and anti-CD31-Alexa Fluor 750.

	FFPE IF
	Reference
PDIA4	ab190348
CD34	IC0115 #3569
CD55	ab13684
CD31	#98025
CD68	sc20060

Table 9. List of primary antibodies (PDIA4 and key cell markers) and their references used for FFPE immunofluorescence. FFPE: Formalin-fixed paraffin-embedded; IF: immunofluorescence

On the fourth day, nuclear staining was performed using DAPI (1:1000 dilution) for 30 minutes. Slides were mounted with ProLong Diamond Antifade Mountant and stored at 4 °C until imaging. Image acquisition was conducted using the Leica Stellaris 8 confocal microscope, and subsequent localization and semi-quantification of the proteins of interest were performed using QuPath software [279].

5.6. Cell culture and treatments

Synovial tissues were prospectively collected by orthopedic surgeons at CHU Liège from patients undergoing total knee arthroplasty for OA. These consisted of residual surgical material. FLS were isolated from synovial membrane via enzymatic digestion with collagenase overnight at 37 °C. The resulting cell suspensions were filtered and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Cells between passages 3 and 7 were used for experiments. Synovial tissue samples for FLS isolation were obtained from 52 patients (38% women, 62% men), with a median age of 66.5 years (range: 54–84) and a median BMI of 34.5 kg/m² (range: 19.05–38.27) and 11 out of 52 patients had diabetes (21.2%). Cells were exposed to an ER stress with Tunicamycin (Tm; 1 µg/mL; Sigma Aldrich, 11089659), a pro-inflammatory or pro-fibrotic process with respectively TNF- α (10 ng/mL; Peprotech, UK) or TGF- β 1 (10 ng/mL; Thermo Fisher, PHG9214) for 1, 3 or 7 days. For supernatants, FBS 1% was used instead of FBS 10%. Proteins extracts and/or culture supernatants were collected (Figure 18). These stimulation time points were defined based on preliminary data from our laboratory on OA FLS [89,201] and supported by the literature, showing time-dependent induction of ER stress markers (e.g., CHOP) [280], pro-inflammatory cytokines (e.g., IL-1 β , IL-6) [89,281], and pro-fibrotic markers (e.g., α SMA, fibronectin) [89,201].

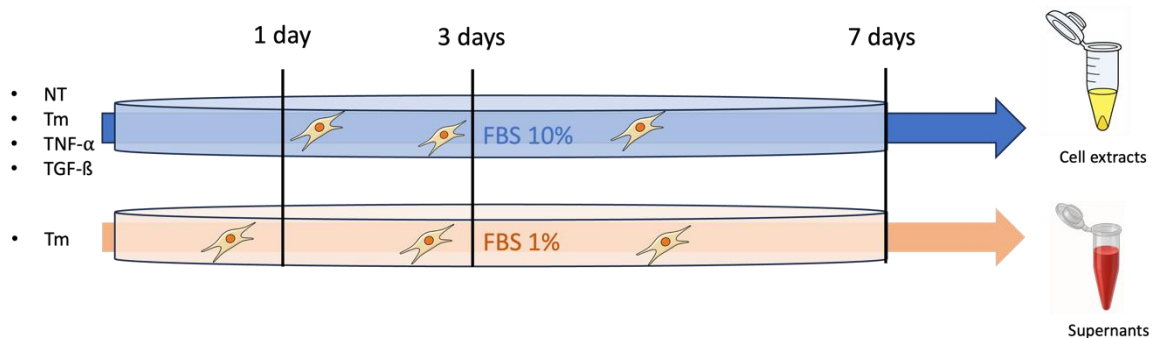


Figure 18. Schematic representation of FLS stimulations (NT, Tm, TNF- α or TGF- β), including stimulation timing and subsequent applications. NT: non-treated; Tm: tunicamycin; TNF- α : tumor necrosis factor- α ; TGF- β : transforming growth factor β ; FLS: fibroblast-like synoviocytes; FBS: fetal bovine serum.

The pharmacological inhibitors of IRE1 α (4 μ 8C- 100 µM; MedChemExpress, HY-19707), ATF6 (Ceapin-A7 0.5µM MedChemExpress HY-108434) or PERK (PERK-IN-2- 0.01µM; MedChemExpress, HY-135220) pathways (UPR), were applied 4 h before a 1-day stimulation

with Tunicamycin. Diméthylsulfoxyde (DMSO) was used on controls. Protein extracts were collected for downstream analysis (Figure 19).

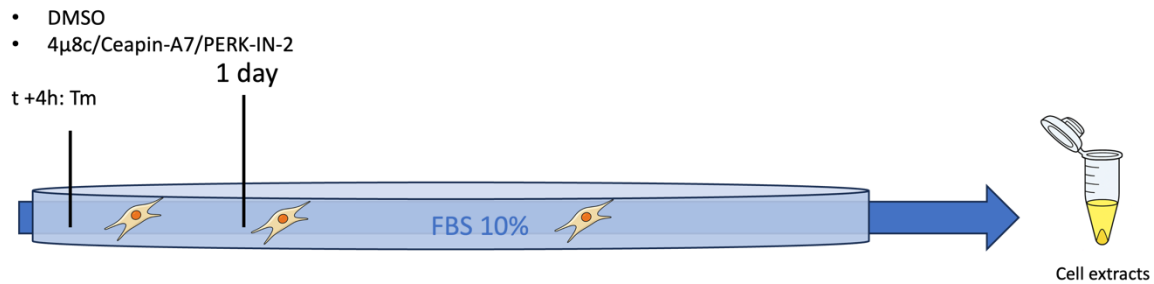


Figure 19. Schematic representation of FLS stimulations (DMSO or 4μ8c/Ceapin-17/PERK-IN-2 with Tm), including stimulation timing and subsequent applications. FLS: fibroblast-like synoviocytes; DMSO: diméthylsulfoxyde; Tm: tunicamycin; FBS: fetal bovine serum.

5.7. Immunofluorescence on FLS

FLS were cultured on glass coverslips and exposed to tunicamycin (Tm; 1 μg/mL) for 3 days. Cells were fixed in 4% paraformaldehyde for 10 minutes, followed by permeabilization and blocking in PBS containing 1% BSA, 5% FBS, and 0.1% Triton X-100 for 30 minutes. Primary antibodies against rabbit anti-CD55 or rabbit anti-CD34 (ab133684, ab81289 respectively) were applied separately overnight at 4 °C. After washing, coverslips were incubated for 1 hour with Alexa Fluor 647-conjugated anti-rabbit secondary antibody (A-21235; Invitrogen). Nuclear counterstaining was performed with DAPI (1:1000), and coverslips were mounted using ProLong Gold Antifade Mountant.

5.8. siRNA transfection

For RNA interference, silencing of HYOU1 (s20632), MANF (s15436), PDIA4 (s225164), DNAJB11 (s28581) (ref. 4427037), or CALR (s115) (ref. 4427038) was achieved by transfecting specific Small Interfering (si)RNAs (Life Technologies) using Lipofectamine reagent (Thermo Fisher Scientific, Cat. No. 11668030) according to the manufacturer's instructions. A non-targeting siRNA (siCtrl; 4390843) served as a negative control. FLS were plated and transfected with siRNA for 24 hours, then stimulated for 3 days with either TGF-β (for fibrosis evaluation or proliferation/viability assays) or TNF-α (for IL-6 quantification). Supernatants and cell extracts were collected in parallel to assess responses and verify effective depletion of ER stress proteins (Figure 20).

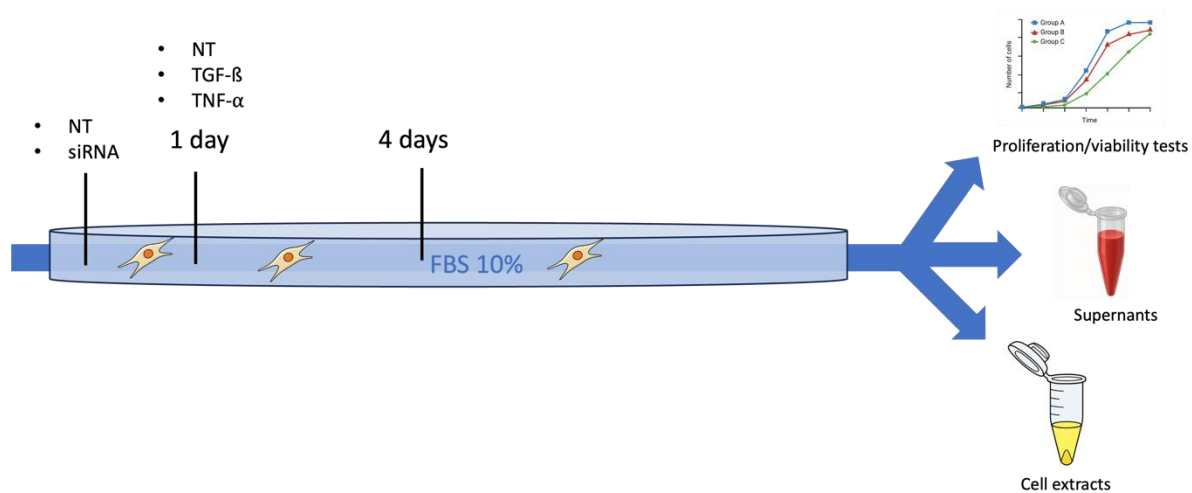


Figure 20. Schematic representation of FLS stimulations (NT or siRNA with or without TGF-β/TNF-α), including stimulation timing and subsequent applications. NT: non treated; TGF-β: transforming growth factor β; TNF-α; FLS: fibroblast-like synoviocytes; siRNA: small interfering RNA; FBS: fetal bovine serum.

5.9. Viability and proliferation assays

FLS were transfected with siRNAs targeting HYOU1, MANF, PDIA4, DNAJB11, CALR, or a control siRNA (siCtrl), then stimulated with TGF-β for 3 days. Cell viability was assessed using the MTS assay (Promega, Madison, Wisconsin, USA) and proliferation using the colorimetric BrdU assay (Abcam), according to the manufacturers' instructions. Absorbance values were corrected by subtracting the background signal measured in DMEM-only wells. Data are therefore presented as background-corrected optical density values rather than percentages of viable or proliferating cells. Negative values may occur following background subtraction and reflect signals close to or below the assay background rather than negative proliferation or viability. BrdU was added approximately 48 hours after TGF-β stimulation, during the final 12–16 hours prior to measurement. All experiments were performed using consistent cell seeding densities and experimental conditions optimized in preliminary experiments to ensure measurements within the dynamic range of the assay. Proliferation and MTS assays were performed separately for HYOU1 and DNAJB11 on the one hand, and for MANF, PDIA4, and CALR on the other, which explains why they are not presented on the same graph, as they were cultured with different concentrations of siCtrl and lipofectamin. Accordingly, their respective depletions were carried out and quantified in parallel but analyzed independently.

5.10. Western blotting

FLS were lysed in RIPA buffer, and proteins from cell lysates were separated by polyacrylamide gel electrophoresis. Proteins were transferred overnight (O/N) onto polyvinylidene difluoride membranes (Millipore, Tullagreen, Ireland) and blocked with 10% milk for 1h at room temperature (RT). Membranes were incubated with primary antibodies (Table 10) for 3 hours at RT, washed, and then exposed to horseradish peroxidase–conjugated secondary antibodies (anti-mouse or anti-rabbit; Cell Signaling Technology) for 45 min at RT. Detection was performed using an enhanced chemiluminescence substrate (ECL; Thermo Scientific, Rockford, USA). GAPDH (#G9545; Sigma) and HSP90 (#13171-1-AP; Proteintech) served as loading controls for intracellular extracts, while Ponceau S stain (CAS-6226795; Sigma Aldrich) was used for supernatants. Band intensities were normalized to the corresponding loading control and quantified using ImageJ (v1.53).

	WB
	Reference
BiP	#3177, Cell Signaling
HYOU1	#ab134944
MANF	#ab67271
PDIA4	#ab155800
GANAB	#ab176349
HSP90B1	#20292, Cell Signaling
TXNDC5	#CF507281, Origen
DNAJB11	#ab224082
LMAN1	#ab125006
ERP29	#ab176573
Calreticulin	#ab92516
CD34	#ab81289
CD55	#ab133684
α -SMA	Clone 1A4, GA611; DAKO
Fibronectin	CS26836S, Cell Signaling
p-IRE1 α	PA1-16927, invitrogen
ATF6	MA5-57851, invitrogen
p-eIF2 α	9721s, Cell Signaling

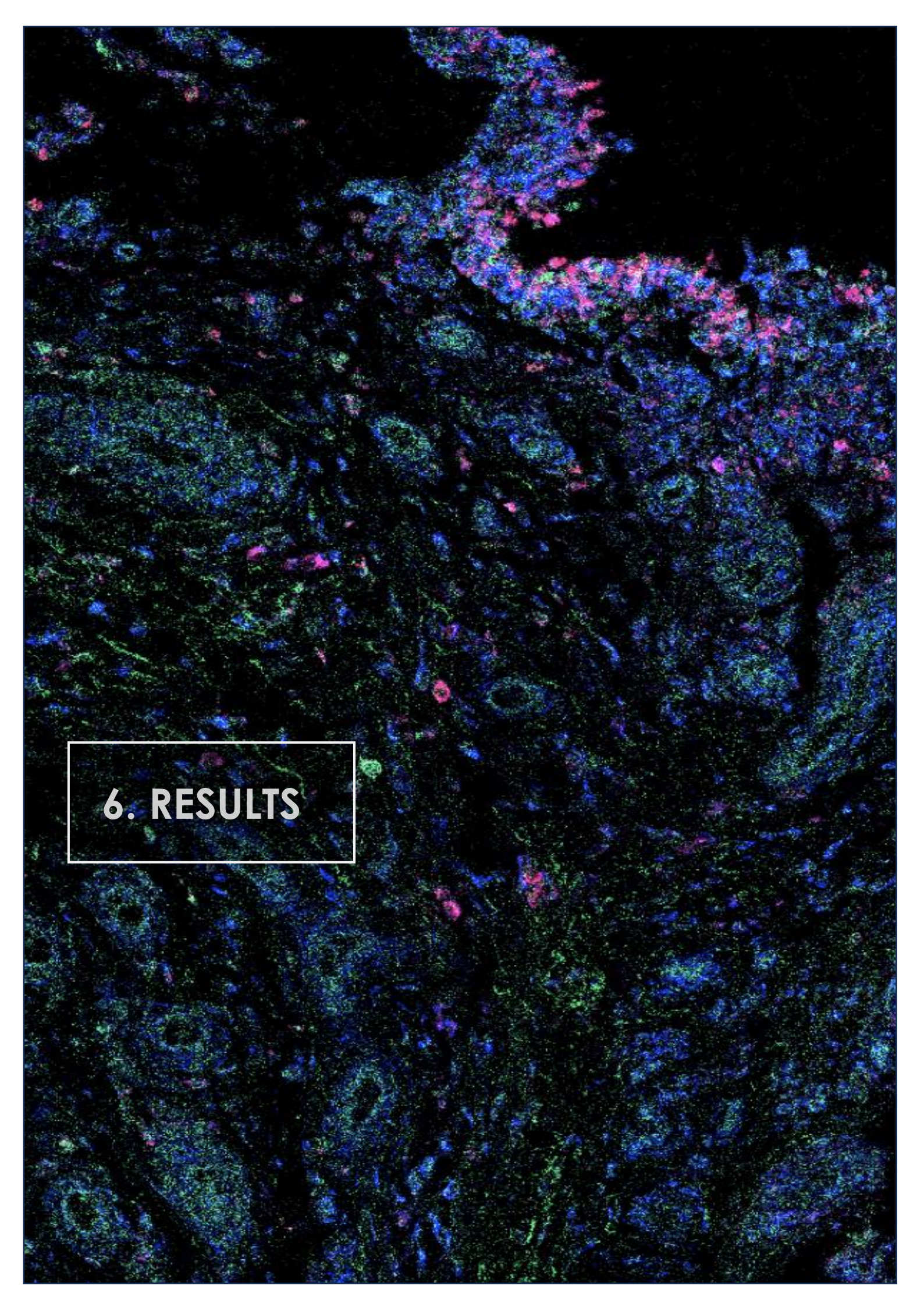
Table 10. List of primary antibodies and their reference used for WB. WB: western blotting.

5.11. Enzyme-Linked Immunosorbent Assay (ELISA)

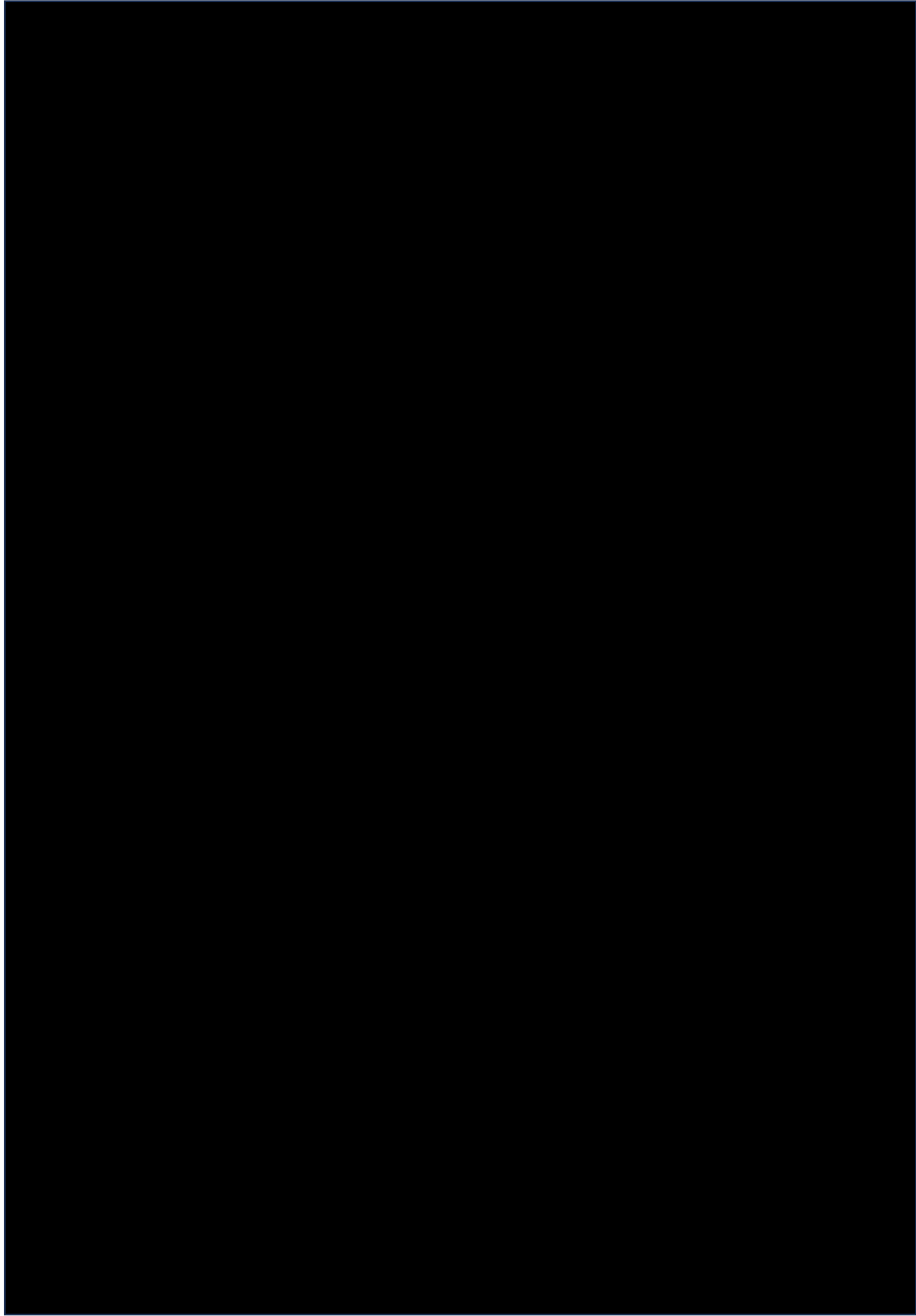
IL-6 levels in cell supernatants were measured using commercially available sandwich ELISA kits (R&D Systems, Minneapolis, MN, USA) following the manufacturer's instructions. The assay detection range was 9.38–600 pg/mL, and all samples were analyzed in duplicate.

5.12. Statistical analysis

Data were analysed with Prism™ software (v9.3.1, GraphPad, Boston, USA) and were considered statistically significant when p-values were <0.05 (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$). The Kolmogorov–Smirnov test was used to assess the distribution. For values that passed the normality test, parametric tests were applied: a paired t-test for two-group comparisons and a paired ANOVA test followed by a Tukey post hoc test for multiple comparisons. For values that did not pass the normality test, non-parametric tests were used. For paired data, the Wilcoxon signed-rank test (two groups) or the Friedman test (more than two groups), followed by Dunn’s post hoc test with appropriate correction for multiple comparisons, was applied. For unpaired data, the Kruskal–Wallis test followed by Dunn’s post hoc test was used for multiple group comparisons.



6. RESULTS



6.1. Distribution and expression levels of endoplasmic reticulum stress proteins in inflamed synovial membranes

6.1.1. Spatial redistribution of ER stress proteins with inflammation in human inflamed synovial membranes

Following our previous LC–MS/MS–based proteomic analysis [13], we identified a set of eleven ER stress proteins—BiP, HYOU1, MANF, PDIA4, GANAB, HSP90B1, TXNDC5, DNAJB11, LMAN1, ERP29, and CALR—in synovial membranes from patients with OA, CPPD, and RA. This mass spectrometry approach provided a semi-quantitative assessment of protein abundance at the tissue level, reflecting the average (“bulk”) expression of ER stress proteins within synovial biopsies, without resolving their spatial distribution. Importantly, the expression levels of these proteins closely correlated with the inflammatory continuum defined by the Tak histological score.

To gain insight into the tissue localization of these ER stress proteins and overcome the limitations of the proteomic approach, their expression was subsequently assessed by IHC on synovial biopsies from the same patient cohort (Cohort 1). Expression patterns were analyzed across OA, CPPD, and RA synovial samples, thus representing a broad and continuous range of inflammatory severity. Protein staining was assessed using a semi-quantitative, optical density–based color scale, classifying cells as highly positive (> 0.6 , red), moderately positive ($0.4\text{--}0.6$, orange), weakly positive ($0.2\text{--}0.4$, yellow), or negative (< 0.2 , blue). This approach allowed simultaneous evaluation of relative protein abundance and spatial localization within the tissue.

All targeted ER stress proteins were detected, although TXNDC5 consistently displayed lower staining intensity compared to the other proteins. In biopsies with low inflammatory activity (Tak score < 4), ER stress protein expression was predominantly confined to the synovial lining layer. In contrast, in samples with higher inflammatory scores (Tak score ≥ 4), staining extended into the sublining compartment. This progressive redistribution was consistently observed across OA, CPPD, and RA biopsies and closely paralleled the inflammatory gradient (Figure 21).

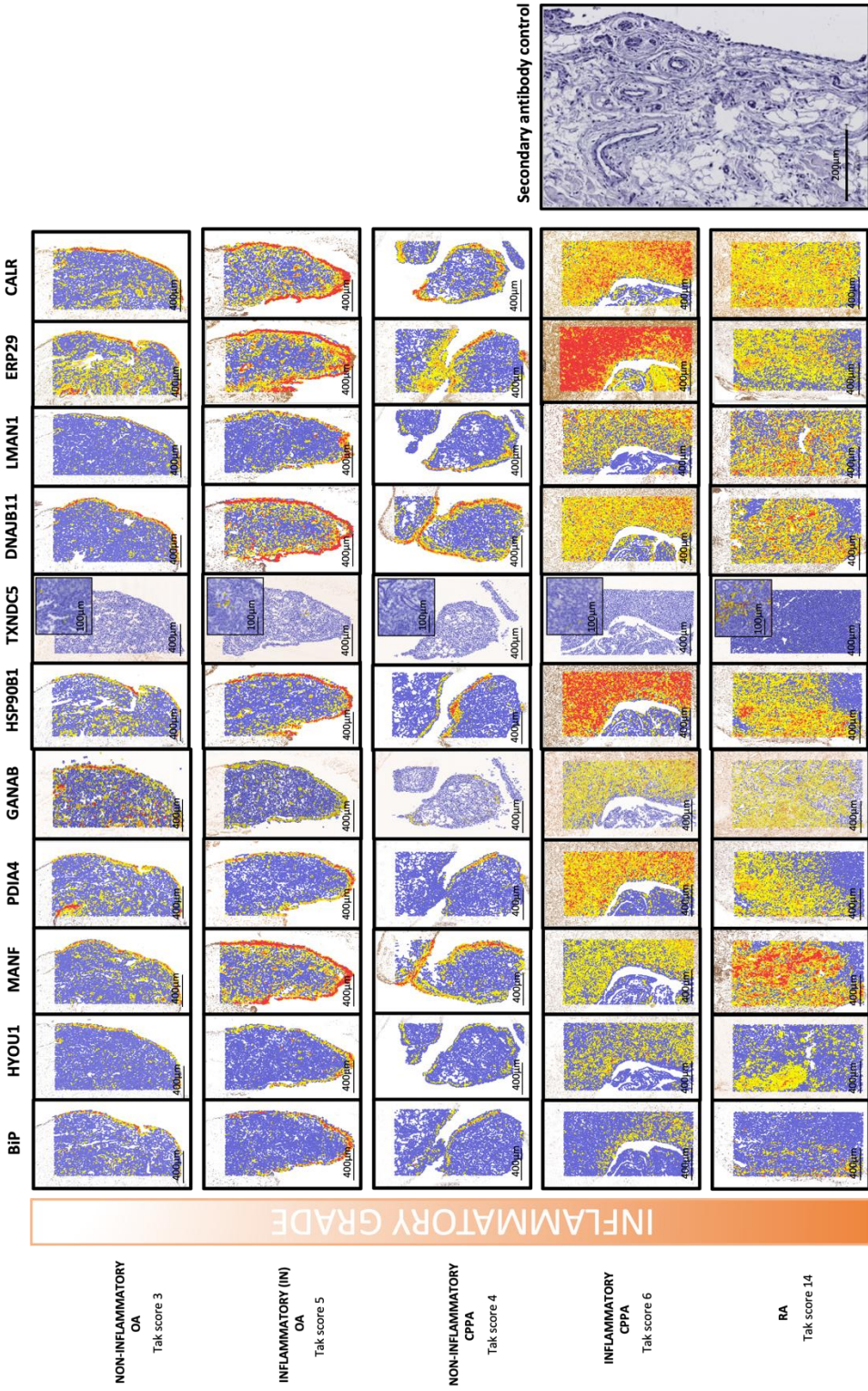


Figure 21. Localization of 11 ER stress proteins in synovial membranes from patients with OA, CPPD, and RA. BiP, HYOU1, MANF, PDIA4, GANAB, HSP90B1, TXNDC5, DNAJB11, LMAN1, ERP29, CALR and secondary antibody control. Immunohistochemistry of the 11 proteins of interest in FFPE synovial membranes from OA (n=2, from 9), CPPD (n=2 from 7), and RA (n=1 from 8) patients (Cohort 1). Highly positive cells (OD > 0.6, red), moderately positive (0.4–0.6, orange), weakly positive (0.2–0.4, yellow), and negative (< 0.2, blue). ER: endoplasmic reticulum; NIN: non-inflammatory; IN: inflammatory; OD: optical density; FFPE: Formalin-fixed paraffin-embedded; OA: osteoarthritis; CPPD: chronic pyrophosphate disease; RA: rheumatoid arthritis.

We next quantified the staining intensity of each protein across OA, CPPD, and RA synovial biopsies. This quantitative analysis revealed that MANF, PDIA4, GANAB, TXNDC5, and CALR were significantly overexpressed in RA compared with OA (Figure 22). In contrast, no statistically significant differences were observed for BiP, HYOU1, HSP90B1, DNAJB11, and LMAN1 (Figure 22).

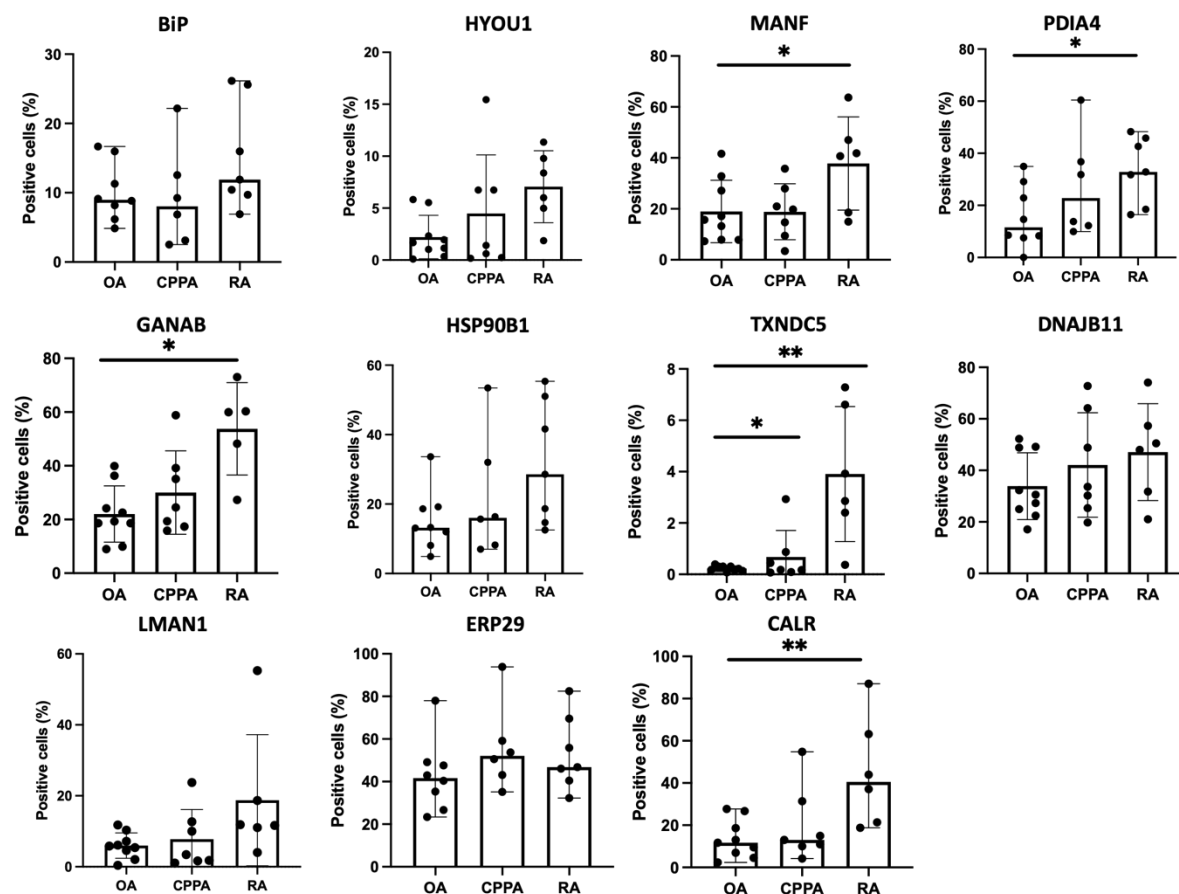


Figure 22. Quantification of 11 ER stress proteins level expression obtained by IHC in synovial membranes from patients with OA (n ≥ 8), CPPA (n ≥ 6), and RA (n ≥ 5). Staining intensity was quantified from immunohistochemistry (IHC) on FFPE synovial sections using QuPath software and expressed as optical density (OD). Data were analyzed with Prism 9 software (GraphPad). Graphs illustrate the median with a 95% confidence interval of the data. Statistical differences were considered for p-values < 0.05 (*p<0.05, **p<0.01, ***p<0.001). Statistical analysis was performed using the nonparametric unpaired Kruskal-Wallis test followed by Dunn's post hoc test for multiple comparisons. ER: endoplasmic reticulum; OD: optical density; FFPE: Formalin-fixed paraffin-embedded; OA: osteoarthritis; CPPA: chronic pyrophosphate arthropathy/disease; RA: rheumatoid arthritis.

The lack of significant differences across disease groups is likely due to the strong overlap in Tak inflammatory scores (Table 6), indicating that ER stress protein expression is more closely associated with inflammatory severity than with disease type. To address this, samples were stratified according to inflammatory status, with non-inflammatory (NIN) and inflammatory

(IN) conditions defined by Tak scores < 4 and ≥ 4 for OA, and < 6 and ≥ 6 for CPPD. In OA and CPPD, a marked difference in protein level expression between NIN and IN conditions was observed for MANF, PDIA4, HSP90B1, DNAJB11, ERP29, and CALR (Figure 23), with a similar trend for HYOU1 and GANAB in CPPD only.

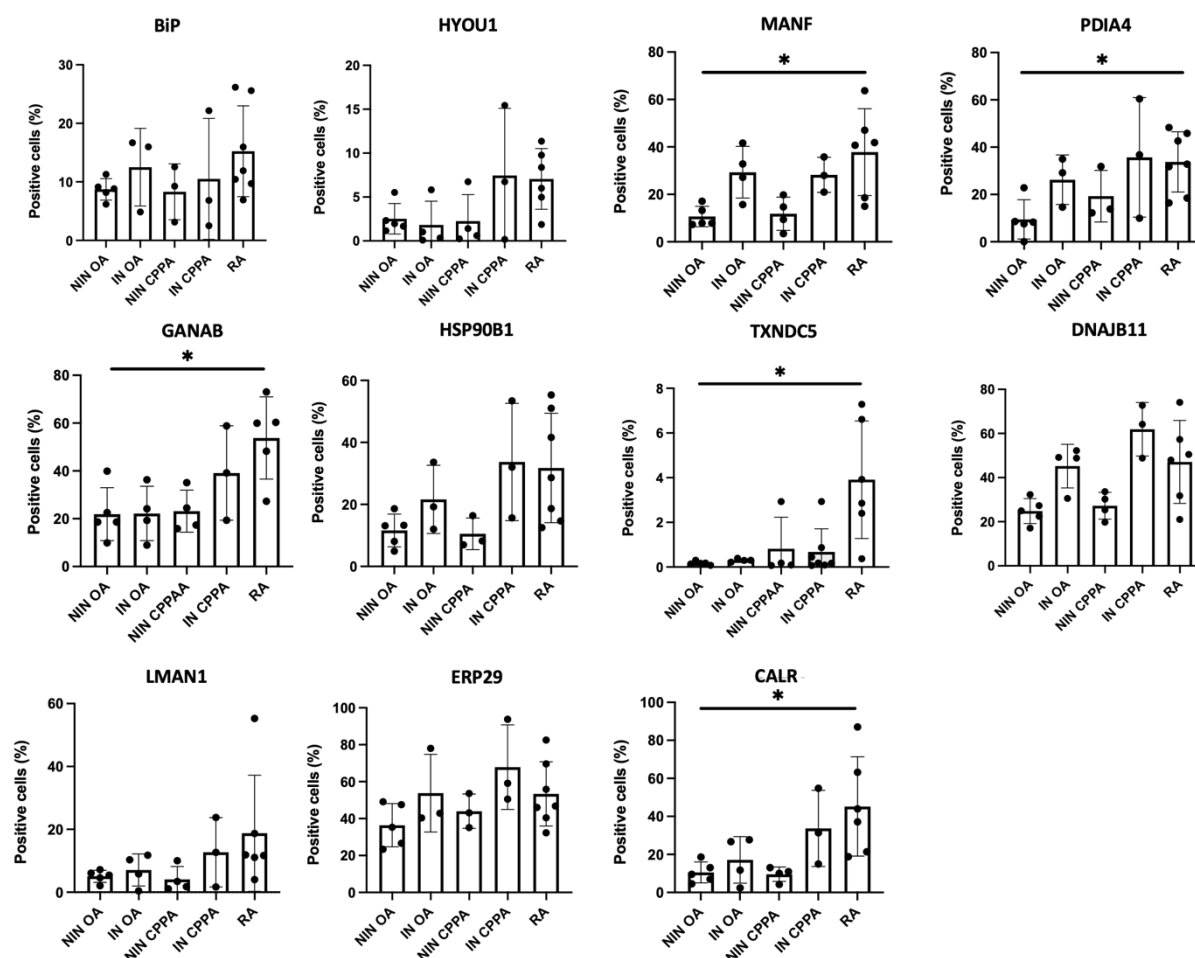


Figure 23. Expression levels of 11 ER stress proteins in synovial membranes from patients with non-inflammatory (NIN) or inflammatory (IN) OA ($n \geq 8$), non-inflammatory or inflammatory CPPA ($n \geq 6$), and RA ($n \geq 5$). Quantification of protein expression was performed using QuPath on FFPE synovial membrane samples from NIN OA, IN OA, NIN CPPA, IN CPPA, and RA patients. Data were analyzed with Prism 9 (GraphPad). Statistical significance was defined as $p < 0.05$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) using a non-parametric, unpaired Kruskal–Wallis test followed by Dunn’s multiple comparison post-hoc test. FFPE: Formalin-fixed paraffin-embedded ; OA: osteoarthritis; CPPA: chronic pyrophosphate arthropathy/disease; RA: rheumatoid arthritis.

The correlation analysis between ER stress-related protein expression levels and the Tak score showed that most proteins were positively correlated with synovitis severity. Indeed, among statistically significant correlations ($p < 0.05$), the lowest observed positive Spearman

correlation coefficient was $r = 0.436$. In contrast, BiP, HYOU1, ERP29, and PDIA4 did not show a significant correlation with the Tak score (Figure 24).

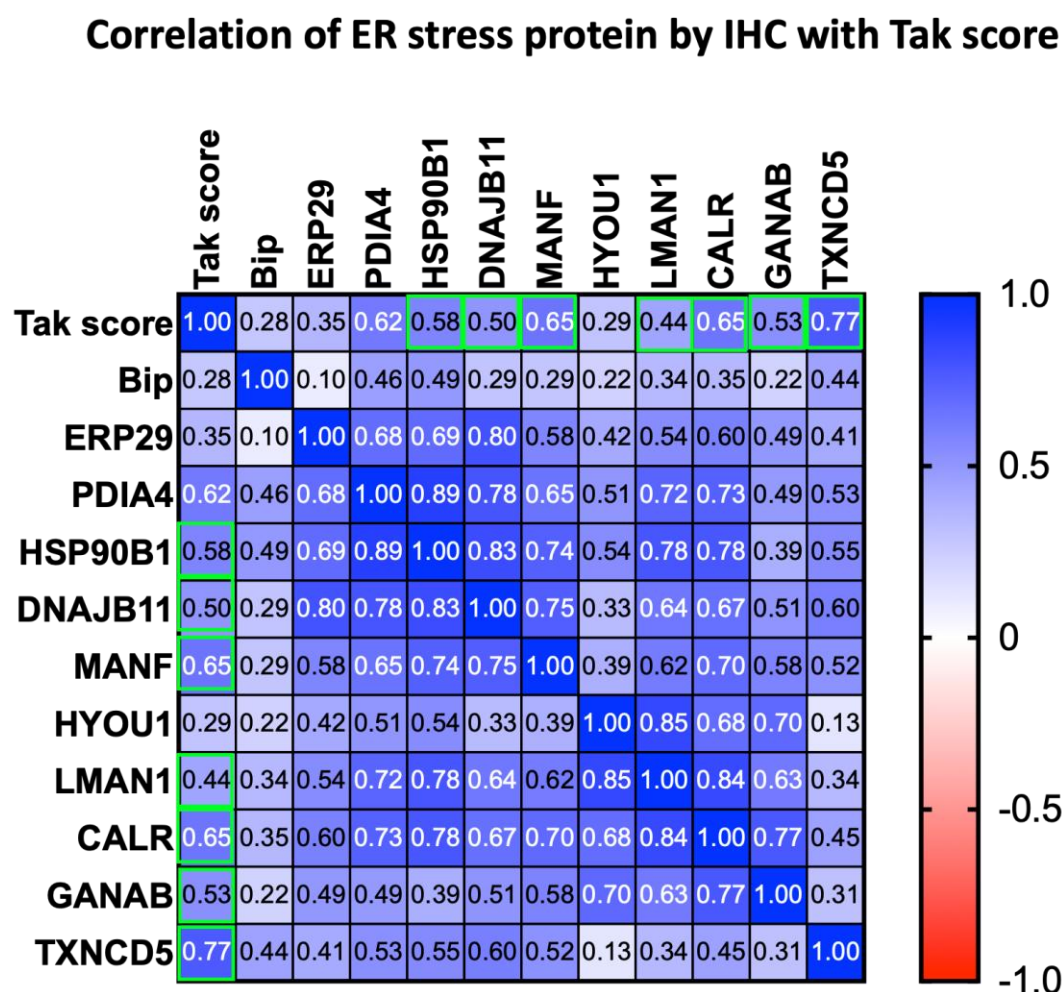


Figure 24. Correlation matrix between ER stress protein expression levels and synovitis severity. Heatmap representing pairwise correlation coefficients between ER stress proteins and the Tak score. Color intensity reflects the strength of the correlation (blue: positive correlation; red: negative correlation). Green boxes highlight statistically significant correlations specifically between the Tak score and ER stress proteins; positive correlations among ER stress proteins themselves are not displayed. Correlations with the histological inflammatory score were assessed using Spearman's rank correlation (correlation matrix). ER: endoplasmic reticulum.

To further extend our spatial analysis, we next used IMC, a high-dimensional imaging technique allowing simultaneous detection of up to 40 proteins on a single tissue section with minimal signal overlap. The selected markers were the 11 ER stress proteins (BiP, HYOU1, MANF, PDIA4, GANAB, HSP90B1, TXNDC5, DNAJB11, LMAN1, ERP29 and CALR) and key synovial cell markers [FLS (CD34 and CD55), MLS (CD68), endothelial cells (CD31), T cells (CD4 and CD3) and B cells (CD20)]. Although this approach was primarily designed to

enable subsequent co-localization analyses with cellular markers of interest, it also served to validate the ER stress protein expression patterns previously observed by IHC in an additional patient cohort (Cohort 2). As performed for IHC, synovial samples were stratified according to inflammatory status defined by the Tak score, and the expression patterns of the eleven ER stress proteins of interest were examined across these groups. In the IMC images, the signal corresponding to the protein of interest is displayed in white, allowing direct visualization of its spatial distribution within the synovial tissue. In line with these observations, analysis of the second cohort, including NIN OA, IN OA, and RA synovial biopsies, confirmed the progressive overexpression of ER stress proteins into the sublining as inflammation progressed (Figure 25). As shown in Figure 25, their expression was restricted to the lining of the Tak 0 biopsy and was predominantly observed in the sublining of the Tak 4 biopsy. It extended to the entire synovium of the Tak 13 biopsy.

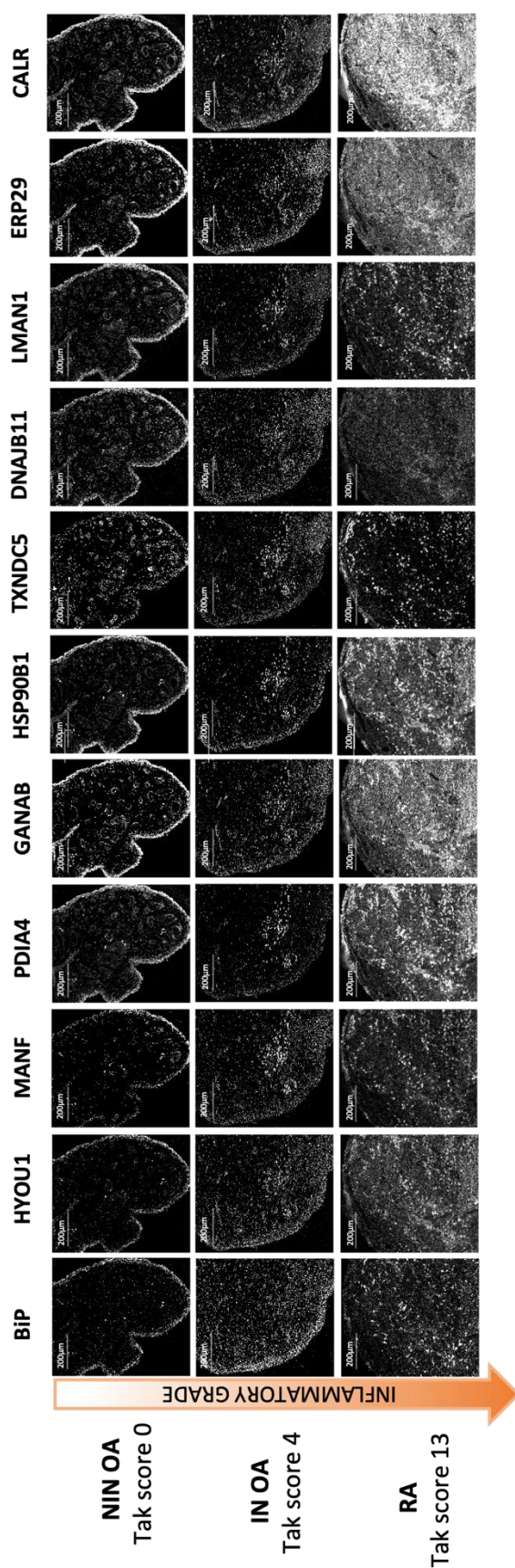


Figure 25. Spatial distribution of ER stress proteins in synovial tissue. A) BiP, HYOU1, MANF, PDIA4, and GANAB. B) HSP90B1, TXNDC5, DNAJB11, LMAN1, ERP29, and CALR. Representative multiparametric images obtained by IMC of synovial sections from patients with OA and RA, illustrating the localization of ER stress proteins. One NIN OA, one IN OA, and one RA sample were selected from six biopsies to depict the inflammatory continuum. ER: endoplasmic reticulum; NIN: non-inflammatory; IN: inflammatory; IMC: imaging mass cytometry; OA: osteoarthritis; RA: rheumatoid arthritis.

This observation was further supported by a semi-supervised clustering analysis of the IMC data. This approach allows cells to be grouped into distinct clusters based on similarities in signal intensity profiles, thereby capturing different levels of protein expression across the tissue. By defining clusters according to the intensity of IMC-derived signals, this method provides a semi-quantitative assessment of ER stress protein expression at the single-cell level, while preserving spatial context. Four clusters (regions 1–4), distinguished by color coding (blue, green, yellow and orange), were defined according to the mean expression levels of ER stress proteins. The column values represent the mean fold change across all ER stress proteins, showing a gradient from low ER protein expression levels in cluster 1 (blue) to high ER protein expression levels in cluster 4 (orange) (Figure 26). The ER stress fold change was calculated as the ratio of each chaperone's expression level in a given region to its average across all the regions in the three biopsies.

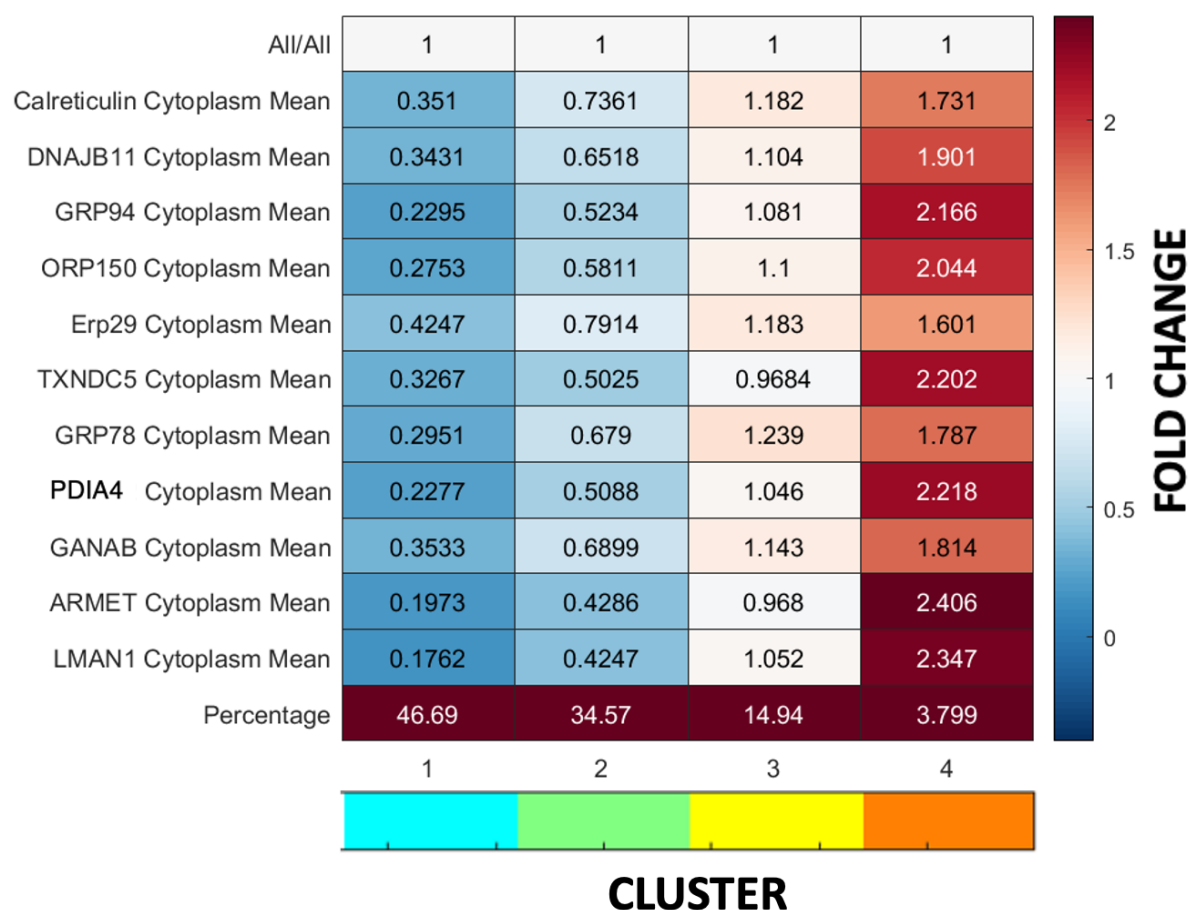


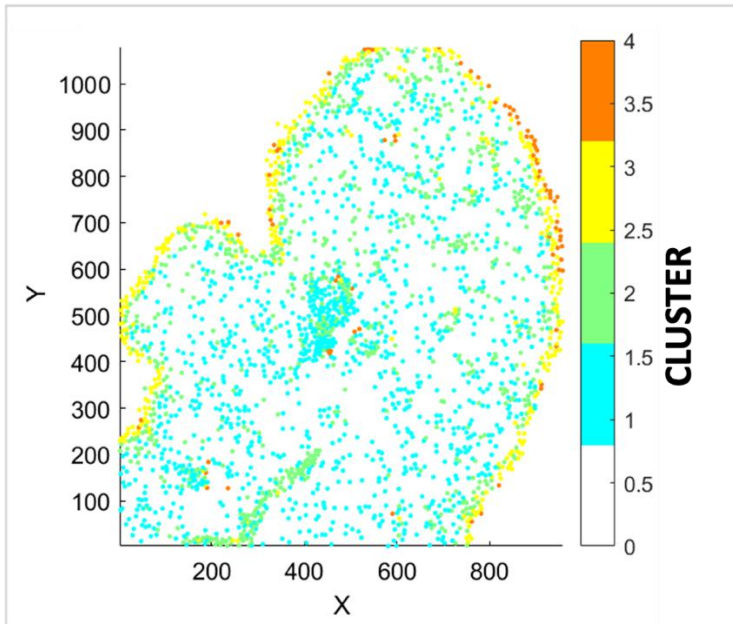
Figure 26. Semi-supervised clustering of ER stress protein expression in synovial tissue. Imaging mass cytometry data were analyzed using a semi-supervised clustering approach to group cells based on cytoplasmic ER stress protein signal intensity. Four clusters were defined, and the heatmap shows the mean fold-change expression of the eleven ER stress proteins, with the blue and orange clusters representing low and high expression of the proteins, respectively. ER: endoplasmic reticulum.

As for the previous analyses, synovial biopsies were stratified according to inflammatory status. The spatial distribution of these four expression levels (referred to as intensity-based clusters), defined by the density-based color scale (blue, green, yellow, and orange) was then mapped onto representative synovial sections from NIN OA, IN OA, and RA samples. The low-expression blue region is predominantly located in the sublining, independently of the inflammatory status. The intermediate-expression clusters green/yellow region are mainly restricted to the lining in both NIN and IN OA. The high-expression orange region, which is of particular interest, extends into the sublining with increasing inflammation. In RA, all expression levels/clusters are diffusely distributed throughout the synovial tissue (Figure 27).

ER stress proteins

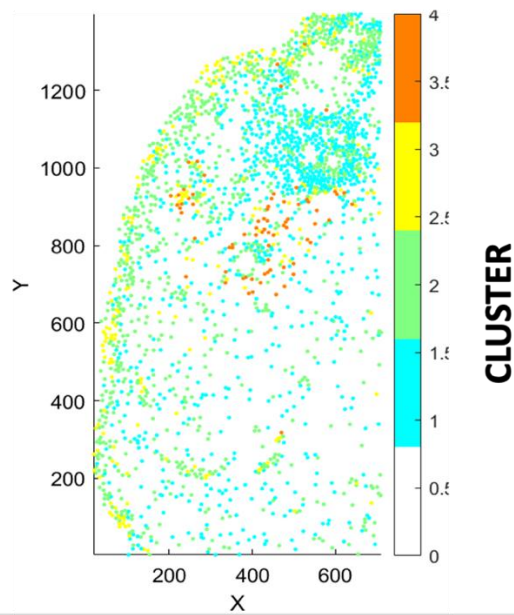
NIN OA

Tak score 0



IN OA

Tak score 4



RA

Tak score 13

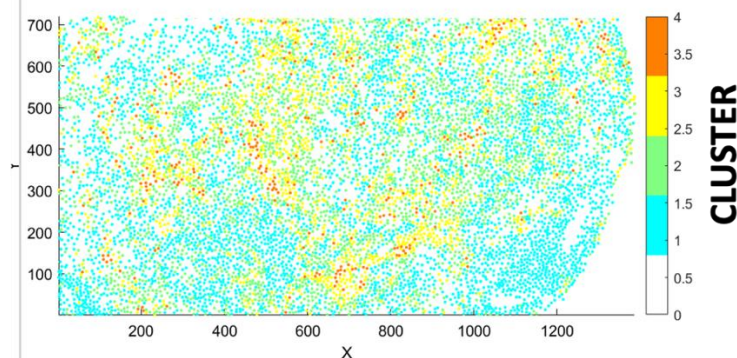


Figure 27. Spatial mapping of ER stress protein clusters in synovial tissue by IMC. The previously established semi-supervised clustering was projected onto representative synovial biopsies from NIN OA, IN OA, and RA. The four color-coded regions correspond to clusters defined by increasing mean ER stress protein expression: blue indicates low expression, green and yellow intermediate expression levels, and orange high expression. ER: endoplasmic reticulum; IMC: imaging mass cytometry; NIN: non-inflammatory; IN: inflammatory; OA: osteoarthritis; RA: rheumatoid arthritis.

6.1.2. Confirmation of ER stress proteins overexpression in Collagenase-Induced Synovitis mouse model

To overcome the limited availability of healthy human synovial tissue, we next turned to murine models. In humans, the collection of NIN synovial membrane is technically challenging due to its thin, non-hyperplastic nature and raises ethical concerns, making it difficult to obtain appropriate healthy controls. Based on antibody compatibility between human and mice, protein expression of BiP, MANF, PDIA4, HSP90B1, DNAJB11, LMAN1 and CALR was investigated by IHC in inflamed synovial membrane of CIS mice compared to control mice. All seven ER stress proteins were detected in all samples, using the same semi-quantitative color-coded scale as that applied to human synovial tissues, with highly positive cells shown in red (optical density > 0.6), moderately positive cells in orange (0.4–0.6), weakly positive cells in yellow (0.2–0.4), and negative cells in blue (< 0.2) (Figure 28A). Immunohistochemistry quantifications showed a significant increase of expression for 6 of them (MANF, PDIA4, HSP90B1, DNAJB11 and LMAN1 and CALR) in CIS compared to controls (Figure 28B).

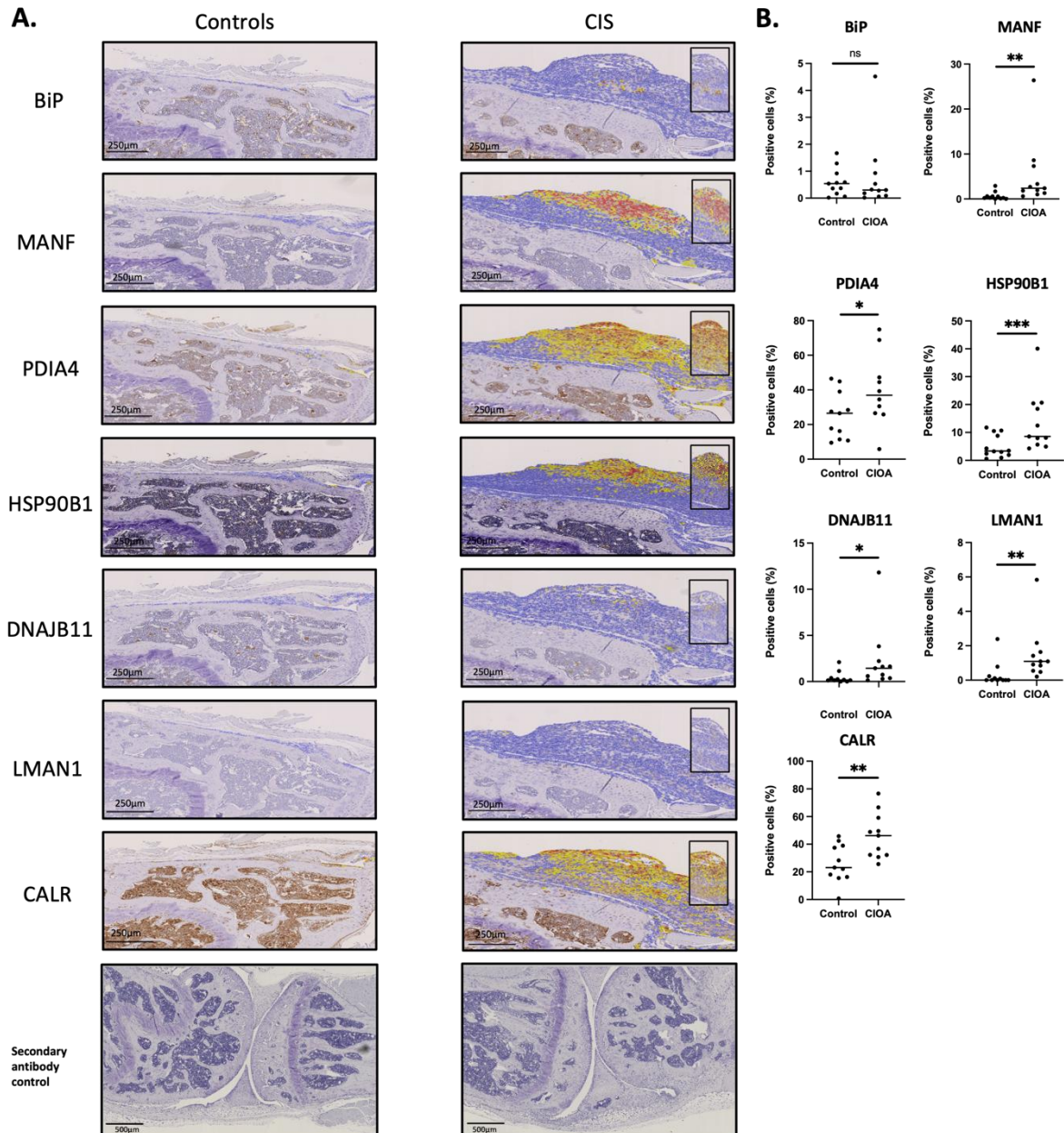


Figure 28. Localization of seven ER stress proteins in synovial membranes from healthy mice and collagenase-induced osteoarthritis (CIS) mouse model. A) Representative IHC images of seven out of the eleven ER stress proteins of interest in FFPE synovial membranes from healthy mouse knees (n=11) and their paired CIS knees (n=11) with their respective secondary antibody control. B) Quantification of protein expression as optical density (OD) values using QuPath. Highly positive cells: OD > 0.6 (red), moderately positive: 0.4–0.6 (orange), weakly positive: 0.2–0.4 (yellow), negative: < 0.2 (blue). Data were analyzed with Prism 9 (GraphPad) using a nonparametric paired t-test (Wilcoxon). Statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. ER: endoplasmic reticulum; IHC: immunohistochemistry; FFPE: Formalin-fixed paraffin-embedded; CIS: collagenase-induced synovitis.

6.1.3. Correlation of ER stress proteins with synovial thickness, inflammatory and/or pro-fibrotic markers in Collagenase-Induced Synovitis mouse model

To further explore the pathological relevance of ER stress protein expression in synovitis, we next investigated whether their levels were associated with key hallmarks of synovial pathology. Specifically, we assessed potential correlations between ER stress protein expression detected by IHC (see above) and markers of inflammation, fibrosis, and synovial hyperplasia. Inflammation was evaluated by the mRNA expression (obtained by RT-qPCR) of the pro-inflammatory cytokines IL-1 β and IL-6, fibrosis by the expression of the myofibroblast marker *Acta2*, and synovial hyperplasia by synovial membrane thickness. The lowest positive Spearman correlation coefficients reaching statistical significance ($p < 0.05$) were $r = 0.818$ for *IL6*, $r = 0.622$ for *IL1 β* , $r = 0.618$ for *Acta2*, and $r = 0.530$ for thickness. These correlations are illustrated in Figure 29. Interestingly, MANF, DNAJB11 and LMAN1 expression levels were positively and significantly correlated with pro-inflammatory cytokines (except DNAJB11 vs IL-6) detected in the synovial membrane of these mice (MANF: $r=0.82$, $p=0.0019$ vs IL-6 and $r=0.78$, $p=0.0038$ vs IL-1 β ; DNAJB11: $r=0.62$, $p=0.0347$ vs IL-1 β ; LMAN1: $r=0.83$, $p=0.0015$ vs IL-6 and $r=0.81$, $p=0.0024$ vs IL-1 β). Further, PDIA4, HSP90B1 and CALR expression levels positively correlated with the fibrotic marker *Acta2* (PDIA4: $r=0.68$, $p=0.0251$, HSP90B1: $r=0.62$, $p=0.0478$ and CALR $r=0.63$, $p=0.0323$). Finally, 5 of the 7 ER stress proteins positively correlated with synovial thickness: MANF ($r=0.60$, $p=0.0032$), HSP90B1 ($r=0.53$, $p=0.0136$), DNAJB11 ($r=0.53$, $p=0.0129$), LMAN1 ($r=0.58$, $p=0.0054$) and CALR ($r=0.56$, $p=0.0067$). BiP did not significantly correlated with any of these parameters.

Correlation of ER stress proteins vs *Il6/Il1b/Acta2*/Thickness

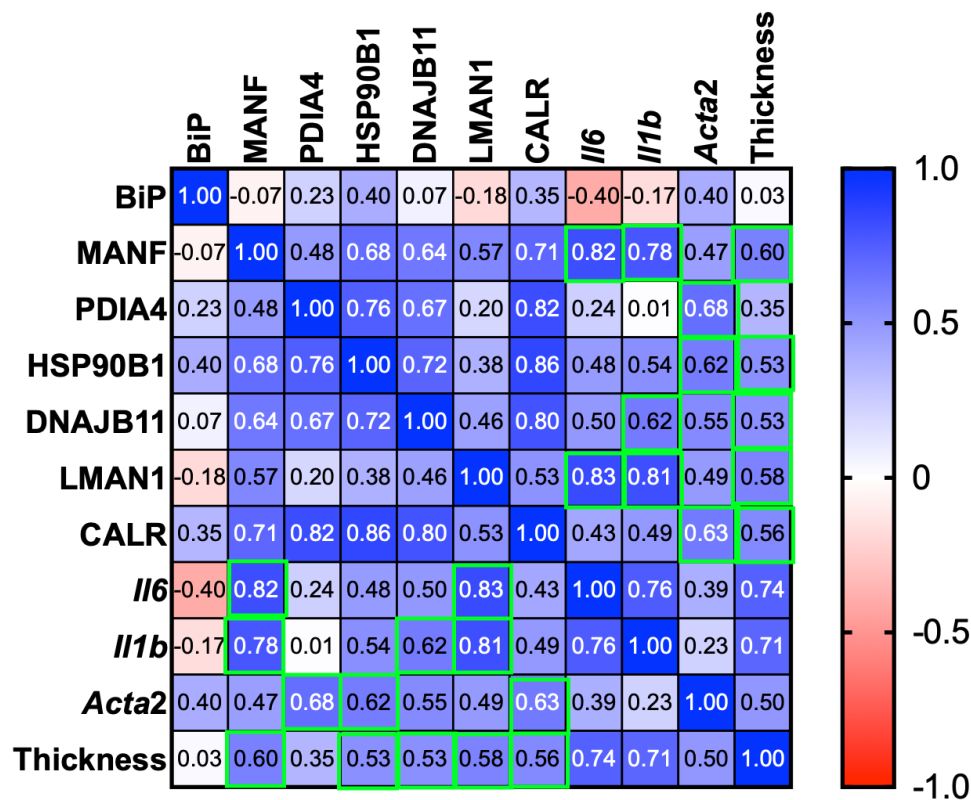


Figure 29. Correlation of ER stress proteins with synovial thickness, inflammatory and/or pro-fibrotic markers in Collagenase-Induced Synovitis mouse model. Spearman's correlation coefficients between IHC-detected protein levels and IL-6, IL-1 β , *Acta2* mRNA expression, as well as synovial thickness. Green boxes indicate statistically significant correlations between ER stress proteins and synovial thickness, inflammatory, or pro-fibrotic markers. ER: endoplasmic reticulum.

- ✓ ER stress proteins redistribute from the lining to the sublining with increasing inflammatory severity.
- ✓ IMC confirms this spatial gradient.
- ✓ CIS model confirms overexpression of ER stress proteins observed in human synovitis.
- ✓ Specific ER stress proteins correlate with inflammation (MANF, DNAJB11, LMAN1), fibrosis (PDIA4, HSP90B1, CALR), and synovial thickness (MANF, HSP90B1, DNAJB11, LMAN1, CALR).

6.2. Coexpression of ER stress proteins with fibroblast-like synoviocytes

6.2.1. Cell populations expressing the ER stress proteins

As described in Section 5.1.1, we previously showed by both IHC and IMC that ER stress proteins are predominantly localized in the lining layer of mildly IN OA synovium (Tak score < 4) and progressively extend into the sublining as inflammation increases (IN OA, Tak score $\geq$ 4). However, at this stage, the identity of the synovial cell populations expressing these ER

stress proteins remained unclear. To address this question, IMC was further exploited to characterize the main cellular compartments of the synovial membrane on the cohort 2.

Key synovial cell populations were identified using CD55 as a marker of lining FLS (red), CD34 as a marker of sublining FLS (yellow), and CD68 as a marker of MLS (blue) (Figure 30). Their spatial distribution was also analyzed across the NIN OA, IN OA, and RA samples. In NIN OA, CD55 and CD68 expression was largely restricted to the lining layer. In contrast, in IN OA, CD55 and CD68 expression remained mainly confined to the lining layer. CD55 expression was largely absent in RA synovium. CD34 displayed a distinct pattern. While already present in the sublining of NIN OA samples, its expression became more prominent and spatially expanded within the sublining in IN OA, closely resembling the distribution of ER stress proteins under inflammatory conditions. In RA, both CD34 and CD68 were broadly dispersed throughout the synovial tissue, consistent with the loss of normal synovial architecture observed in this disease.

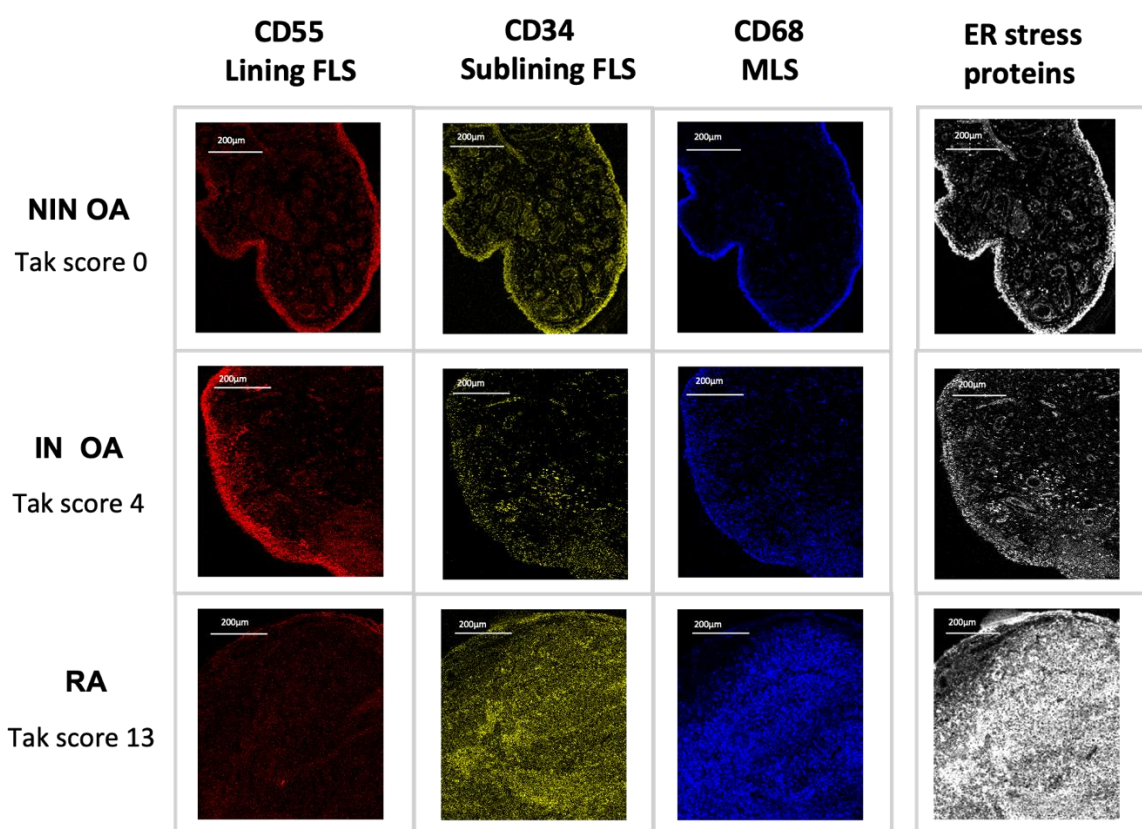


Figure 30. Distribution of FLS and MLS within inflamed synovial membranes. Qualitative analysis (direct detection) of synovial FLS (CD55, red or CD34, yellow), MLS (CD68, blue) and ER stress proteins across NIN and IN OA, and RA. FLS: fibroblast-like synoviocytes; MLS: macrophage-like synoviocytes; IMC: imaging mass cytometry; NIN: non-inflammatory; IN: inflammatory; OA: osteoarthritis; RA: rheumatoid arthritis.

To objectively define marker expression levels, an intensity-based thresholding approach was applied. For CD34, the analysis was restricted to CD34⁺CD31⁻ cells to exclude endothelial cells, allowing the identification of low- and high-expression populations for each marker.

These expression levels were subsequently projected back onto the tissue sections (NIN OA, IN OA and RA) to generate spatial maps of marker distribution (Figure 31). In these maps, low-expression cells are shown in blue, whereas high-expression cells are shown in red. Consistent with the observations described above, CD55 showed strong expression within the lining layer in both NIN and IN OA but was absent in RA synovium. Conversely, CD34 was predominantly localized in the sublining compartment in IN OA and extended throughout the synovial tissue in RA. CD68 displayed a distribution pattern similar to that of CD55 in OA, remaining largely confined to the lining layer, but persisted in RA tissues.

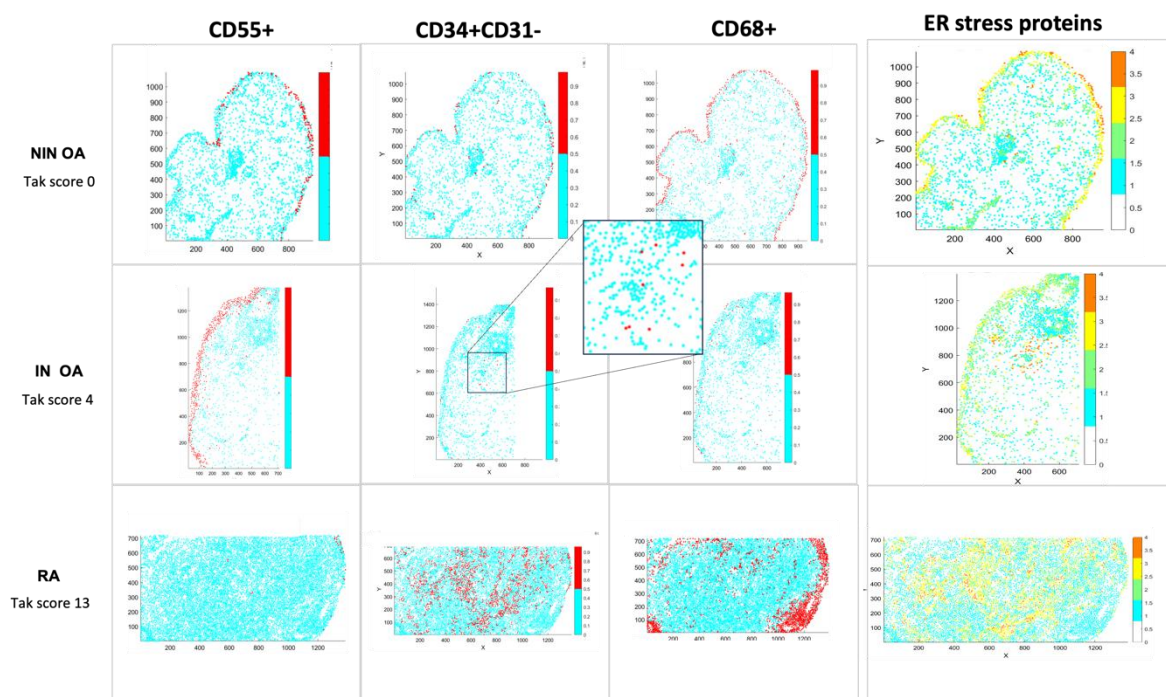


Figure 31. Spatial distribution of FLS and MLS within synovial membranes. IMC-based mapping of CD55⁺ FLS, CD34⁺CD31⁻ FLS, CD68⁺ MLS in NIN and IN OA, and RA. Marker expression levels were defined using an intensity-based thresholding approach (mean + 2 SD after exclusion of outliers beyond 1.5× IQR in OA samples), red dots indicate high-expression cells, whereas low-expression cells are shown in blue. The clustering of ER stress proteins are replaced beside them for clarity. FLS: fibroblast-like synoviocytes; MLS: macrophage-like synoviocytes; IMC: imaging mass cytometry; NIN: non-inflammatory; IN: inflammatory; OA: osteoarthritis; RA: rheumatoid arthritis; SD: standard deviation; IQR: interquartile range.

Using the same analytical framework applied to synovial cellular markers, we next examined lymphocyte populations. T lymphocytes were identified using CD3 (cyan) and CD4 (green), while B cells were detected using CD20 (purple) (Figure 32). Their spatial distribution was analyzed in synovial biopsies from the same cohort (Cohort 2), including NIN OA, IN OA, and RA samples.

For both NIN and IN OA biopsies, CD3⁺, CD4⁺ and CD20⁺ positive cells displayed comparable expression patterns and were predominantly localized within lymphoid aggregates. In addition, a limited CD4 signal was also observed within the lining layer in OA, independently of inflammatory status, likely reflecting CD4 expression by MLS rather than T cells [282,283]. In RA synovium, lymphocyte markers were broadly distributed throughout the tissue.

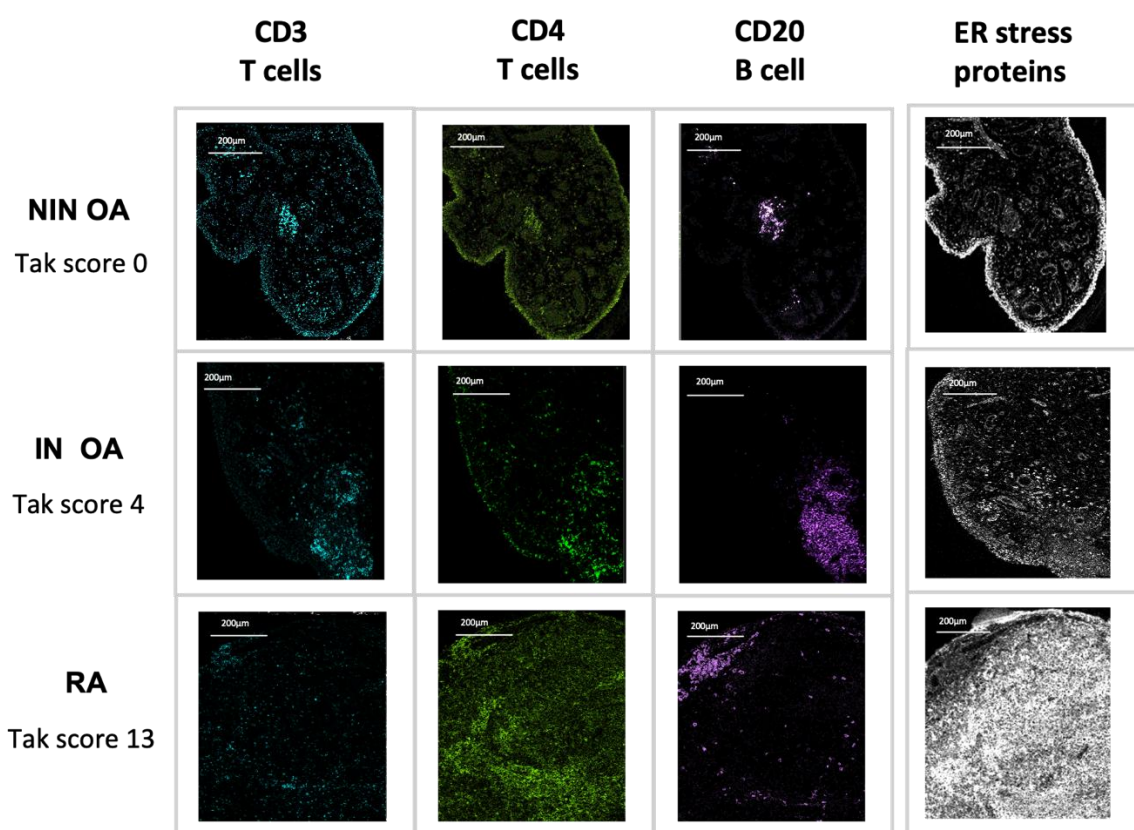


Figure 32. Spatial distribution of lymphocyte markers in synovial tissue analyzed by IMC. Representative synovial biopsies from NIN OA, IN OA, and RA were analyzed by IMC. T lymphocytes were identified using CD3 (cyan) and CD4 (green), while B lymphocytes were detected using CD20 (purple). ER stress proteins are also represented for clarity. IMC: imaging mass cytometry; NIN: non-inflammatory; IN: inflammatory; OA: osteoarthritis; RA: rheumatoid arthritis. ER: endoplasmic reticulum.

To define lymphocyte marker expression levels, an intensity-based thresholding approach was also applied to CD3⁺ and CD4⁺ T cells, as well as CD20⁺ B cells, allowing the identification of low- and high-expression populations for each marker. These were then projected onto synovial tissue sections (Figure 33), with low-expression cells shown in blue and high-expression cells in red.

In both NIN and IN OA, lymphocyte markers were predominantly localized within lymphoid aggregates. In contrast, in RA synovium, CD3⁺, CD4⁺, and CD20⁺ cells displayed a more diffuse distribution throughout the tissue. Unlike FLS and MLS markers, lymphocyte markers did not exhibit a preferential localization to either the lining or sublining compartments.

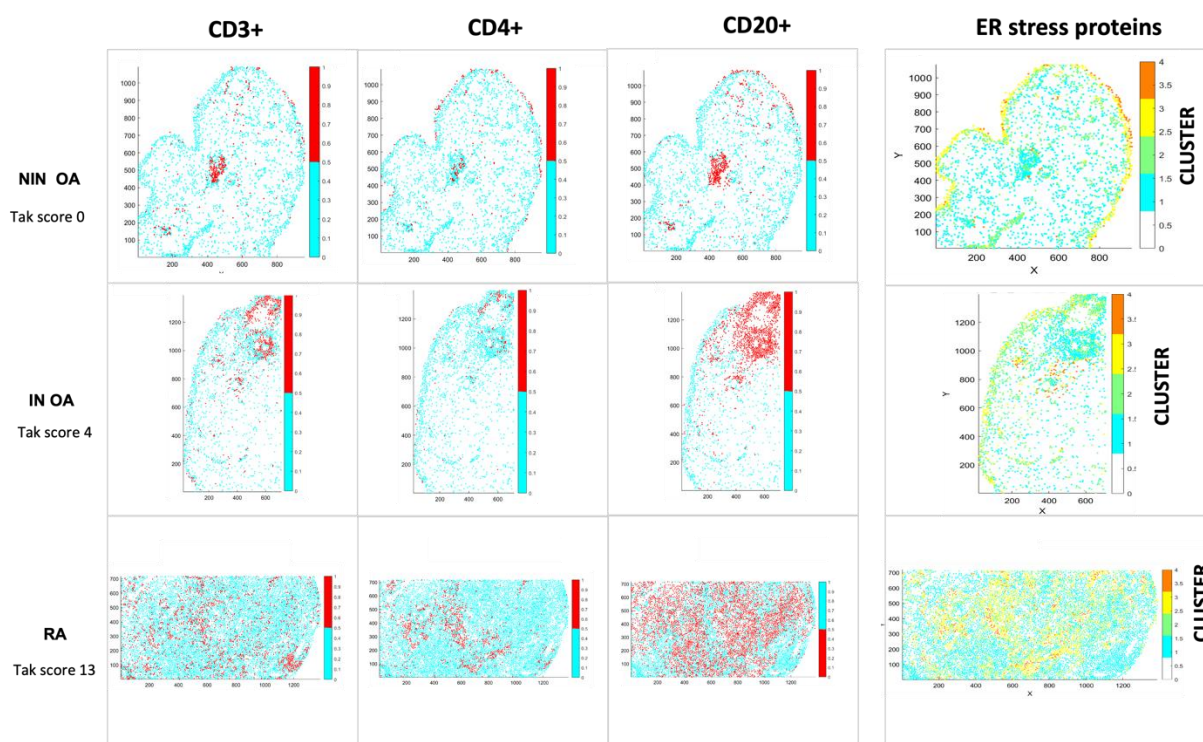


Figure 33. Distribution of lymphocyte markers within (non)-inflamed synovial membranes. IMC-based spatial mapping of CD3⁺ and CD4⁺ T cells and CD20⁺ B cells in NIN OA, IN OA, and RA. Marker expression levels were defined using an intensity-based thresholding approach (mean + 2 SD after exclusion of outliers beyond 1.5× IQR in OA samples). Red dots indicate high-expression cells, whereas low-expression cells are shown in blue. The clustering of ER stress proteins is placed beside them for clarity. ER: endoplasmic reticulum; SD: standard deviation; IQR: interquartile range.

6.2.2. ER stress protein co-expression shifts from lining (CD55⁺ FLS, CD68⁺ MLS) to sublining (CD34⁺ FLS) with increasing inflammation

Following earlier data mapping ER stress proteins and synovial cell populations (CD55⁺, CD34⁺CD31⁻, and CD68⁺), we used once again IMC (Cohort 2) to assess their potential co-expression with these cell markers and identify possible interactions within the synovial membrane. Since all ER stress proteins displayed similar spatial patterns (Figure 34), PDIA4 was chosen, in the qualitative analysis, as a representative marker for clarity.

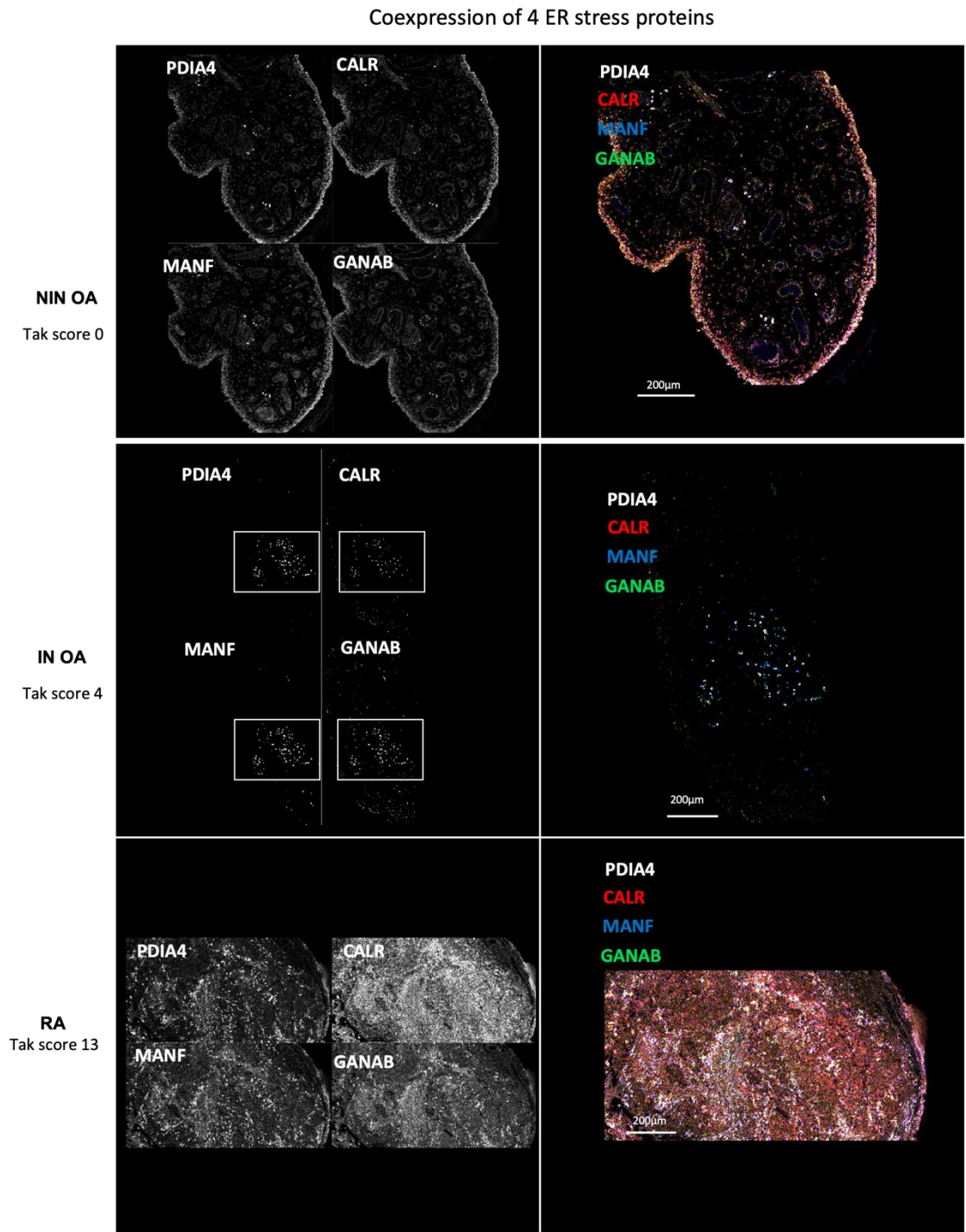


Figure 34. Distribution and coexpression of ER stress proteins within (non)-inflamed synovial membranes. Qualitative analysis (direct detection) of PDIA4 (white), CALR (red), MANF (blue) and GANAB (green) coexpression by IMC across NIN and IN OA and RA, with their coexpression in overlay images with white squares enclosing the infiltrated marker. ER: endoplasmic reticulum; IMC: imaging mass cytometry; NIN: non-inflammatory; IN: inflammatory; OA: osteoarthritis; RA: rheumatoid arthritis.

As shown in Figure 35, in NIN OA (Tak <4), ER stress proteins were predominantly localized in the lining layer, where they were co-expressed with CD55⁺ FLS and with CD68⁺ MLS, though less abundantly. In this compartment, CD34⁺ FLS were nearly absent in NIN OA. As inflammation progressed (IN OA), ER stress proteins infiltrating the sublining, were co-expressed with CD34⁺CD31⁻ FLS, while CD55 and CD68 remained in the lining. In RA, CD55 expression was lost. CD34, CD68, and the ER stress proteins were diffusely distributed throughout the synovium, which had lost its normal architecture. The right panel highlighted the combined markers (also with CD31) among the different inflammatory stage.

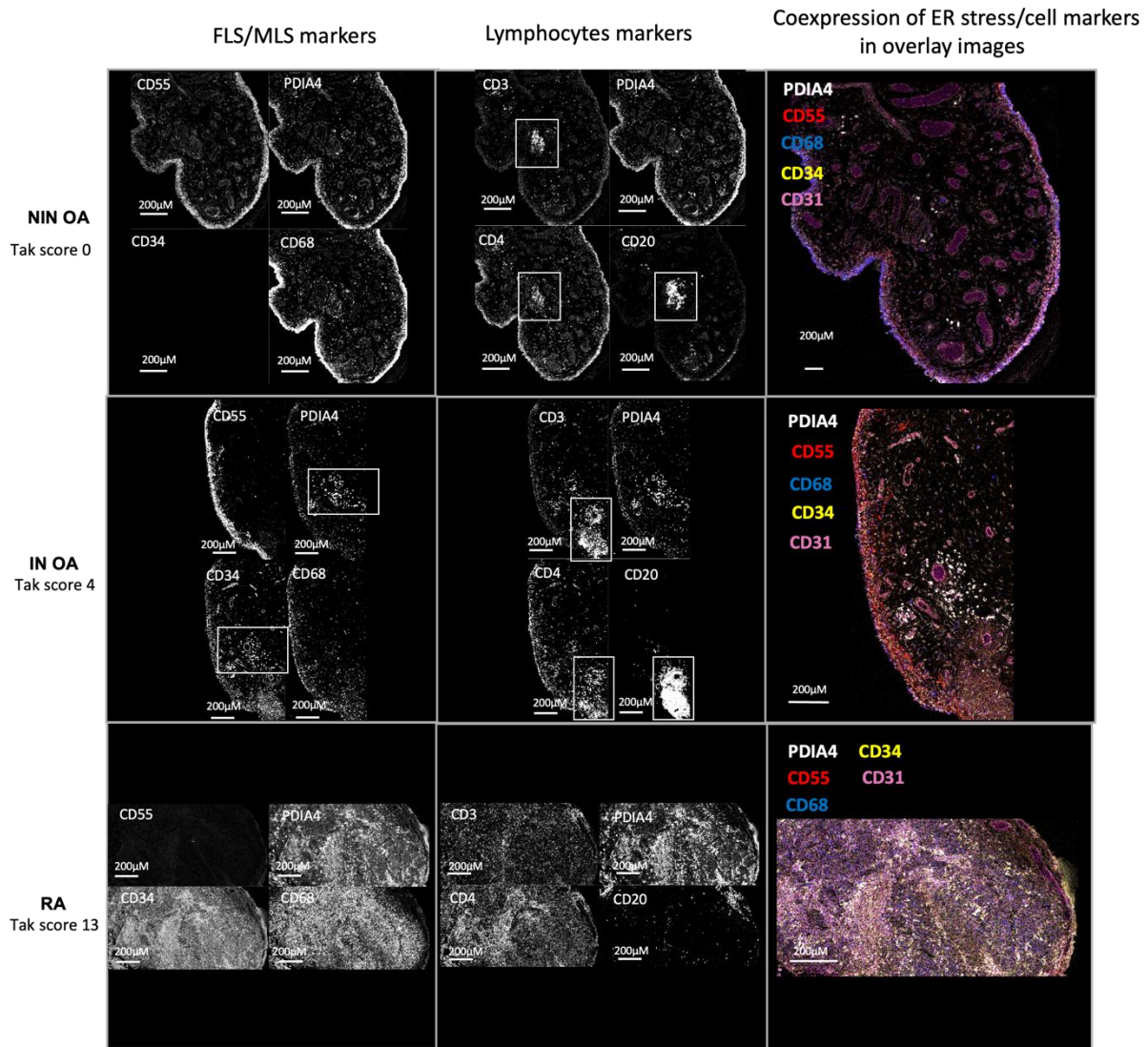


Figure 35. Co-expression of PDIA4 with FLS/MLS in the synovial membrane of NIN OA, IN OA and RA. Qualitative analysis (direct detection) by IMC of PDIA4 and synovial cell (FLS, MLS and lymphocytes) markers with their coexpression in overlay images (also with CD31) with white squares enclosing the infiltrated marker. FLS: fibroblast-like synoviocytes; IMC: imaging mass cytometry; MLS: macrophage-like synoviocytes; NIN: non-inflammatory; IN: inflammatory; OA: osteoarthritis; RA: rheumatoid arthritis.

These co-expression patterns were further supported by a supervised clustering analysis assessing the co-expression between each cellular marker of interest (FLS or MLS markers) and the entire panel of ER stress proteins. This analysis identified two distinct clusters: one characterized by low co-expression and low fold-change values (green cluster / low-intensity regions), and a second displaying high co-expression with elevated fold-change values (blue cluster / high-intensity regions) (Figure 36). These clusters were subsequently projected onto tissue spatial coordinates to visualize their distribution within synovial samples (Figure 37). Fold-change values were calculated as the ratio of the expression level of each ER stress protein

at the single-cell level within a given region to its mean expression across all regions and across the three analyzed biopsies.

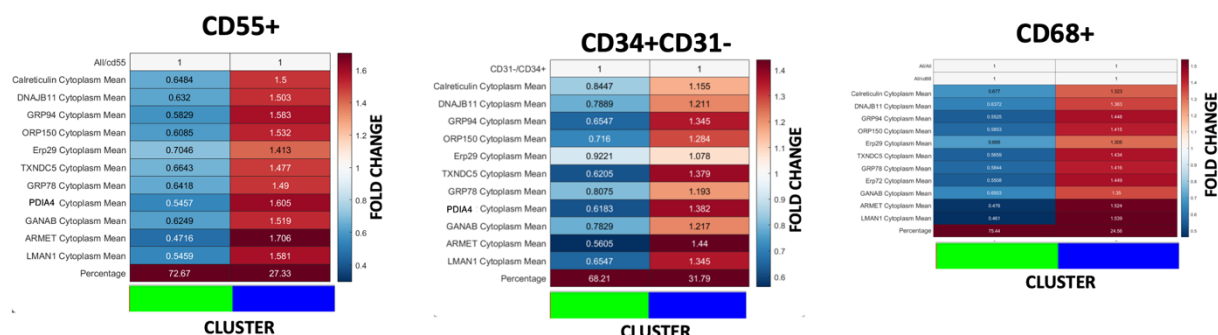


Figure 36. Supervised clustering analysis of ER stress protein co-expression with synovial cellular markers. Imaging mass cytometry data were analyzed using a supervised clustering approach to assess the co-expression between synovial cellular markers (FLS and MLS markers) and the panel of ER stress proteins. For each cellular marker population, two regions were defined based on ER stress protein co-expression levels. Green regions indicate low co-expression, whereas blue regions indicate high co-expression. Spatial clustering was performed in CytoMAP (v1.4.21) using a Self-Organizing Map (SOM) algorithm with nearest-neighbor context, with the optimal number of clusters determined by the Davies–Bouldin index. ER: endoplasmic reticulum; FLS: fibroblast-like synoviocytes; MLS: macrophage-like synoviocytes

Spatial representation of the supervised clustering analysis further refined the cellular context of ER stress protein expression (Figure 37). In NIN OA, the high co-expression cluster (blue cluster), identified by the supervised analysis, revealed a strong colocalization with CD55⁺ FLS and CD68⁺ MLS within the lining layer, while minimal co-expression was observed with CD34⁺CD31⁻ sublining FLS. In IN OA, whereas ER stress proteins are known to infiltrate the sublining compartment (Section 6.1.1), spatial projection of this cluster showed that their expression preferentially co-localized with CD34⁺CD31⁻ FLS. In RA, ER stress proteins associated with the high co-expression cluster were co-expressed with both CD34⁺CD31⁻ FLS and CD68⁺ MLS and were diffusely distributed throughout the synovial tissue. In brief, across inflammatory grades, ER stress protein expression was consistently associated with FLS, with a shift from CD55⁺ lining FLS in NIN OA to CD34⁺CD31⁻ sublining FLS in IN OA and RA.

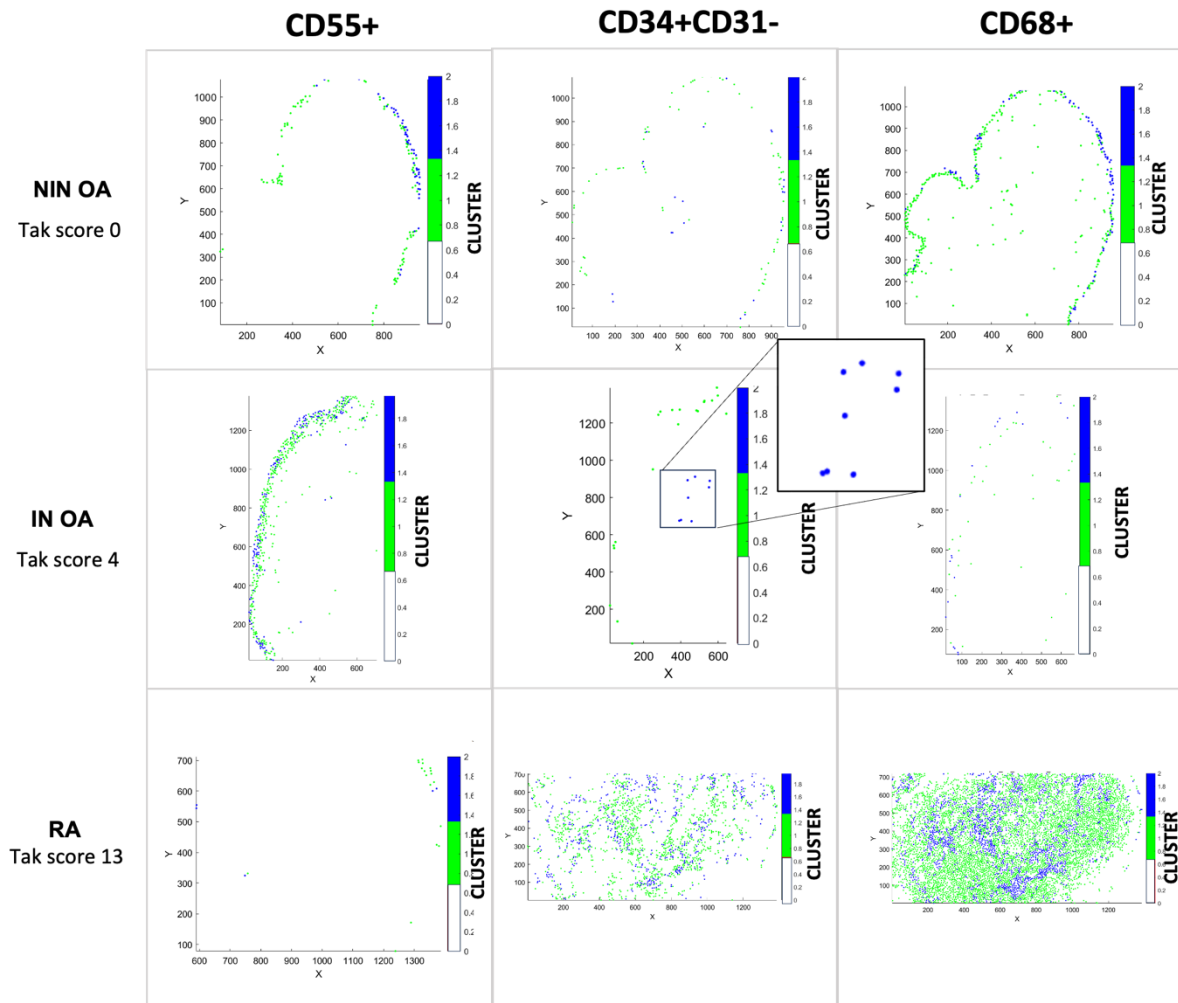


Figure 37. Spatial mapping of supervised clustering of ER stress protein co-expression with synovial cell subsets. ER stress protein expression was first clustered using a supervised approach based on co-expression with FLS and MLS markers. The resulting clusters were then projected onto tissue coordinates to visualize their spatial distribution in NIN OA, IN OA, and RA samples. Blue dots indicate regions with high ER stress protein co-expression within specific cellular subsets, while green dots represent lower levels of co-expression. ER: endoplasmic reticulum; FLS: fibroblast-like synoviocytes; MLS: macrophage-like synoviocytes; NIN: non-inflammatory; IN: inflammatory; OA: osteoarthritis; RA: rheumatoid arthritis.

6.2.3. Confirmation of the coexpression of ER stress proteins with FLS in a larger cohort using immunofluorescence.

Due to the high cost of IMC and the resulting limited cohort size, we sought to validate our findings in a larger patient population. Therefore, co-expression patterns were confirmed by IF in an extended cohort of 25 OA patients (Cohort 3 – CHU Liège). For clarity and technical feasibility, and because simultaneous IF detection of all eleven ER stress proteins together with cellular markers was not possible due to antibody species incompatibilities, PDIA4 was selected as a representative ER stress protein for these validation experiments.

In Figure 38, four OA synovial biopsies representing progressively increasing inflammatory grades were selected. The lining and sublining compartments were identified, and the expression of PDIA4 together with key cellular markers was examined across these inflammatory stages. PDIA4 is shown in white, while cellular markers correspond to those used in the IMC approach: CD55 marking lining FLS (red), CD68 identifying MLS (green), CD34 labeling sublining FLS (yellow), and CD31 used to exclude endothelial cells (purple). Nuclei were counterstained with DAPI (blue). The co-expression of the ER stress protein PDIA4 with FLS markers was confirmed by the overlap of their respective fluorescence signals (Figure 38). In NIN OA (Tak 3), we observed strong co-expression of PDIA4, with CD55⁺ or CD68⁺ cells within a well-preserved lining layer (Figure 38A). This coexpression persisted at a slightly higher inflammatory grade, at which stage the lining layer showed early hyperplasia with the formation of villous-like structures (Tak 5; Figure 38B). As inflammation progressed, the distribution shifted. At Tak 6, limited PDIA4 co-expression with CD55⁺ or CD68⁺ cells remained in the lining. Meanwhile, PDIA4 expression was already detected in the sublining and co-expressed with CD34⁺CD31⁻ FLS (Figure 38C). By Tak 7, the lining had nearly disappeared and the sublining was dominated by CD34⁺CD31⁻PDIA4⁺ FLS (Figure 37D).

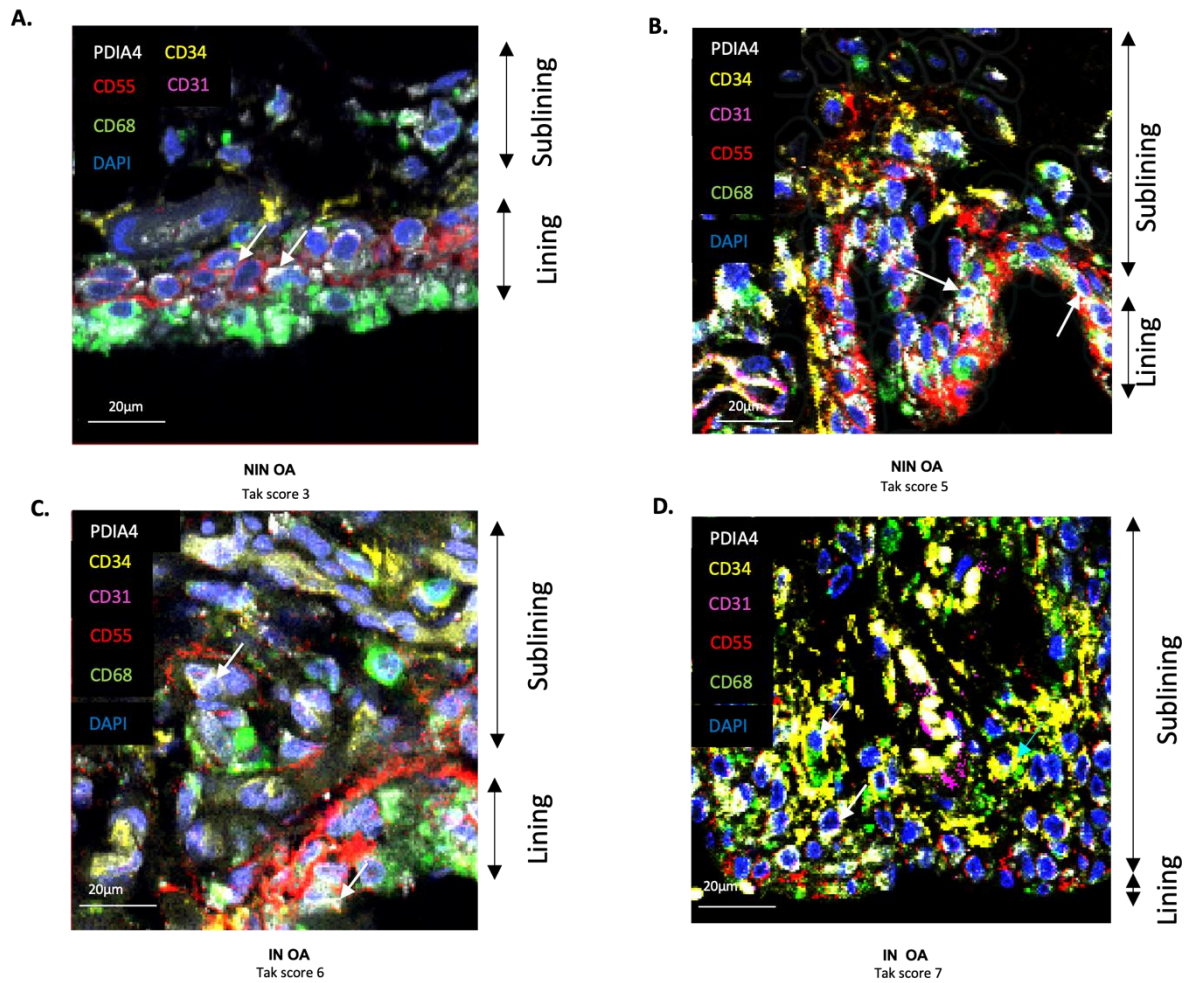


Figure 38. Validation of PDIA4 (ER stress protein) co-expression with FLS in an extended OA cohort by IF (n = 25; Cohort 3). Immunofluorescence staining of PDIA4 (white), CD55⁺ (red), CD68⁺ (green), CD34⁺ (yellow), CD31⁻ (pink), and nuclei (DAPI, blue) in A) NIN (Tak 3), B) intermediate (Tak 5), C) IN (Tak 6) and D) IN (Tak 7) OA synovial samples. White arrows indicate PDIA4 expression within CD55⁺, CD68⁺ or CD34⁺CD31⁻ cells. n: number of patients; ER: endoplasmic reticulum; FLS: fibroblast-like synoviocytes; OA: osteoarthritis; IF: immunofluorescence; NIN: non-inflammatory; IN: inflammatory.

We next quantified the analytical observations described above by performing a systematic quantification of PDIA4 expression across synovial cell populations (Figure 39). For this purpose, a uniform threshold was defined for each cell marker to classify individual cells as PDIA4-positive or PDIA4-negative, and this threshold was applied identically across all tissue sections. Quantification was performed separately in the lining and sublining compartments to assess compartment-specific changes associated with inflammation. Synovial samples were stratified into three inflammatory groups based on the Tak score (Tak 0–3, Tak 4–5, and Tak $\geq$ 6). Within each inflammatory category, the relative proportions of PDIA4-expressing synovial cell subsets were determined. These included the classical CD68⁺ MLS, CD55⁺ lining FLS,

CD34⁺CD31⁻ sublining FLS, as well as an intermediate CD55⁺CD34⁺CD31⁻ FLS population. Importantly, proportions were calculated relative to the total number of cells within these marker-defined populations (i.e. CD68⁺, CD55⁺, CD34⁺CD31⁻, and CD55⁺CD34⁺CD31⁻ cells), irrespective of PDIA4 expression. In parallel, the combined fraction of PDIA4-negative cells across these phenotypes was quantified.

In the lining layer (Figure 39A), % of PDIA4⁺CD55⁺ fibroblasts (red) was higher in non-inflamed OA (30%) and decreased under inflammatory conditions (20%). In contrast, % of PDIA4⁺CD68⁺ macrophages (green) increased with inflammation, rising from 17% in NIN OA to 28% in IN OA. The most pronounced change was observed for PDIA4⁺CD34⁺CD31⁻ FLS, whose proportion (yellow) increased markedly from low levels in NIN OA (7%) to become predominant in IN OA (34%).

In the sublining compartment (Figure 39B), % of PDIA4⁺CD55⁺ cells remained low and relatively stable (14-15%) across inflammatory conditions. CD68⁺PDIA4⁺ cells showed a moderate increase in their relative proportion within the analyzed cell compartment with inflammation (from 11% to 17%). In contrast, CD34⁺CD31⁻PDIA4⁺ fibroblasts displayed the most pronounced shift, increasing from 9% in NIN OA to 31% in IN OA.

Across both compartments, the proportion of PDIA4-negative cells progressively declined with increasing inflammation. In the sublining, this reflects a redistribution of PDIA4 expression across cell types, with CD34⁺CD31⁻ fibroblasts emerging as the dominant contributors to the PDIA4-positive pool.

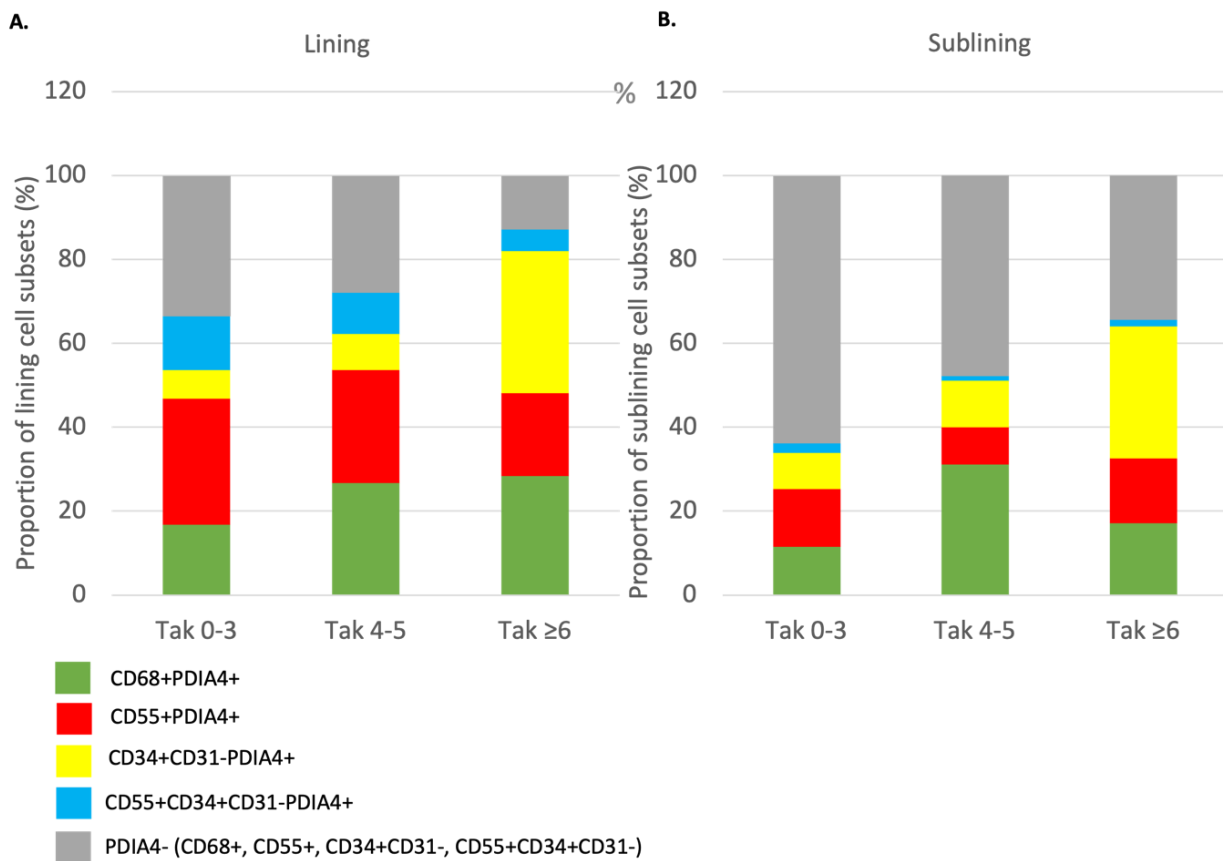


Figure 39. Validation of PDIA4 (ER stress protein) co-expression with FLS in an extended OA cohort by IF (n = 25; Cohort 3). Panels showing the proportion of CD68⁺, CD55⁺, CD34⁺CD31⁻, or CD55⁺CD34⁺CD31⁻ cells expressing or not PDIA4 among total CD68⁺, CD55⁺, CD34⁺CD31⁻ or CD55⁺CD34⁺CD31⁻ cells in the A) lining and B) sublining, stratified by Tak score. n: number of patients; ER: endoplasmic reticulum; FLS: fibroblast-like synoviocytes; OA: osteoarthritis; IF: immunofluorescence.

Next, we examined whether CD55⁺ and CD34⁺ cells represent distinct FLS populations within the synovial membrane or whether CD34⁺ cells can arise from a phenotypic transition of CD55⁺ cells. Co-staining revealed a CD55⁺CD34⁺CD31⁻ subset (Figure 39).

To further explore the relationship between lining and sublining fibroblast populations, we performed a correlation analysis between their relative abundances. For each sample, the proportion of CD55⁺PDIA4⁺ lining FLS (quantified within manually annotated lining regions; y-axis) and CD34⁺CD31⁻PDIA4⁺ sublining FLS (quantified within annotated sublining regions; x-axis) was calculated relative to the total fibroblast population, defined as the sum of CD55⁺, CD55⁺CD34⁺CD31⁻, and CD34⁺CD31⁻ cells, irrespective of PDIA4 expression. A significant inverse correlation was observed (Spearman, $r = -0.43$, $p = 0.03$) (Figure 40A). In contrast, no such correlation was observed for PDIA4-negative FLS populations (Figure 40B).

These findings suggest that PDIA4 expression may be associated with a shift between lining and sublining fibroblast phenotypes during synovial inflammation.

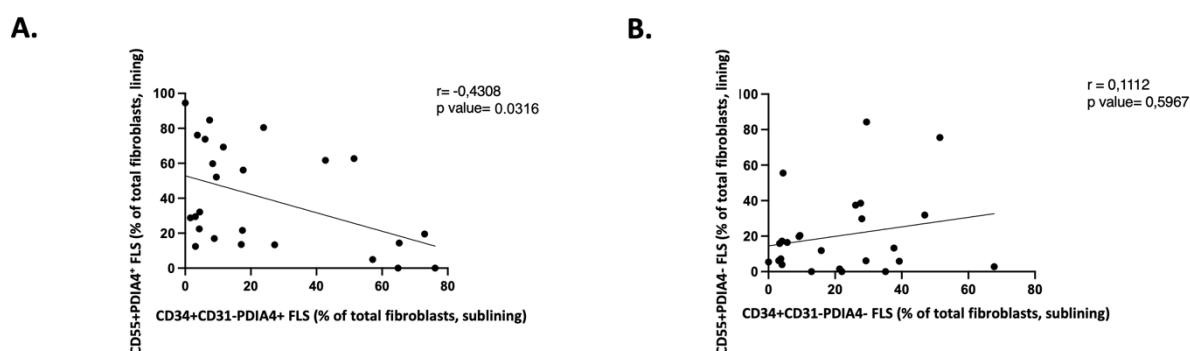


Figure 40. Correlation between PDIA4 expression in lining and sublining fibroblast populations. Spearman correlation analysis showing the relationship between the proportion of CD55⁺ lining FLS and CD34⁺CD31⁻ sublining FLS expressing PDIA4 (A) or not expressing PDIA4 (B). CD55⁺ lining FLS and CD34⁺CD31⁻ sublining FLS were quantified within manually annotated lining and sublining regions, respectively. Proportions are expressed relative to the total fibroblast population, defined as CD55⁺, CD55⁺CD34⁺CD31⁻, and CD34⁺CD31⁻ cells, irrespective of PDIA4 expression. Each point represents one biopsy. The regression line illustrates the relationship between the two fibroblast populations.

- ✓ By IMC, ER stress proteins are mainly expressed by CD55⁺ FLS and CD68⁺ MLS in the lining in NIN OA, and shift to CD34⁺CD31⁻ FLS in the sublining as inflammation increases.
- ✓ Findings are confirmed by IF in an extended OA cohort (n=25); quantification shows expansion of CD34⁺PDIA4⁺ FLS with increasing inflammatory grades.
- ✓ A transitional CD55⁺CD34⁺ subset and inverse correlation suggest that PDIA4 may be involved in fibroblast compartment remodeling.

6.3. ER stress proteins expression and secretion in FLS under ER stress, inflammatory, or pro-fibrotic conditions

6.3.1. Confirmation of CD55 and CD34 expression in synovial fibroblast-like cells by WB & IF

We further pursued in vitro studies based on the co-expression of ER stress proteins with FLS cell type. Human synovial fibroblasts used for these experiments had been previously characterized by flow cytometry in our laboratory and, after three passages in culture, were negative for HLA-DR, CD80, CD45, as well as CD3 and CD31, confirming the absence of immune and endothelial cell contamination [284]. In this research, we first confirmed that our primary FLS did express CD55 and/or CD34 expression by WB (Figure 41). Under TNF- α stimulation, among the eight patients analyzed, six displayed a double-positive CD55⁺CD34⁺ profile, while two patients expressed only CD55. Following tunicamycin treatment, seven

patients were CD55⁺CD34⁺, whereas one patient was CD55⁺ only. Notably, this patient was different from those lacking CD34 expression under TNF- α stimulation. Both markers were detectable at baseline and showed no change following ER stress induction (tunicamycin) or inflammatory stimulation (TNF- α). However, it is important to note that western blot analysis reflects the overall “bulk” protein expression across the entire cell population and does not allow determination of the proportion of CD55⁺CD34⁻, CD55⁻CD34⁺, or CD55⁺CD34⁺ cells. Moreover, due to technical incompatibility between the antibodies used, co-detection at the single-cell level was not feasible in this study. Accurate quantification of these subpopulations would require flow cytometry (FACS)-based analysis.

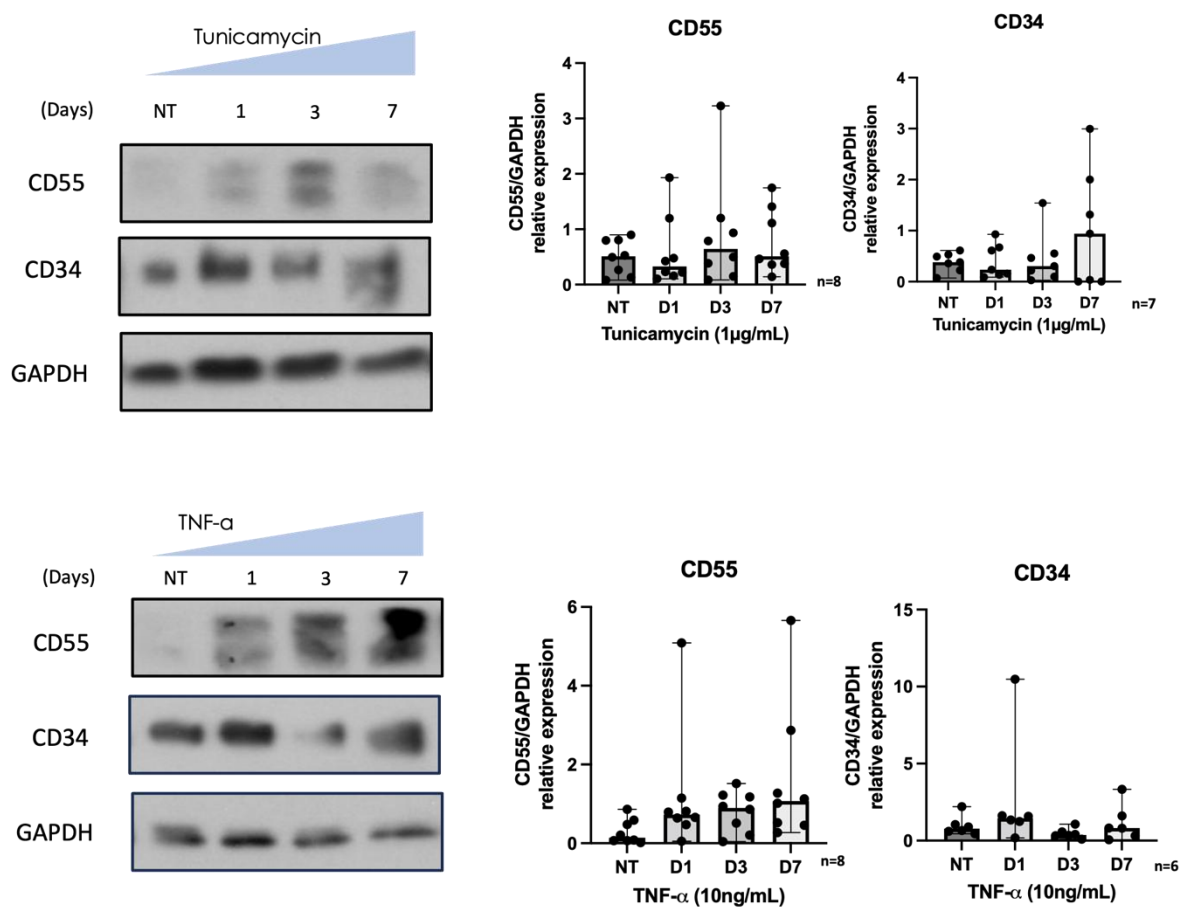


Figure 41. Expression of CD55 and CD34 in OA FLS by WB. Representative WB images and corresponding semi-quantification of FLS stimulated for 1, 3, or 7 days with tunicamycin (1 $\mu\text{g/mL}$) or TNF- α (10 ng/mL) to induce ER stress or a pro-inflammatory response, respectively. OA: osteoarthritis; FLS: fibroblast-like synoviocytes; WB: western blotting; TNF- α : tumor-necrosis factor α ; ER: endoplasmic reticulum.

IF was then used to detect synovial fibroblast markers in their native conformation within intact cells. CD55 showed a nuclear and perinuclear distribution while CD34 showed a peri-

membranous and intracellular distribution, consistent with membrane-associated proteins and with FLS activation under tunicamycin-induced ER stress (Figure 42). Although co-localization of CD55, CD34, and PDIA4 would have been informative, this was not feasible due to antibody species incompatibilities.

Tunicamycin (3 days)

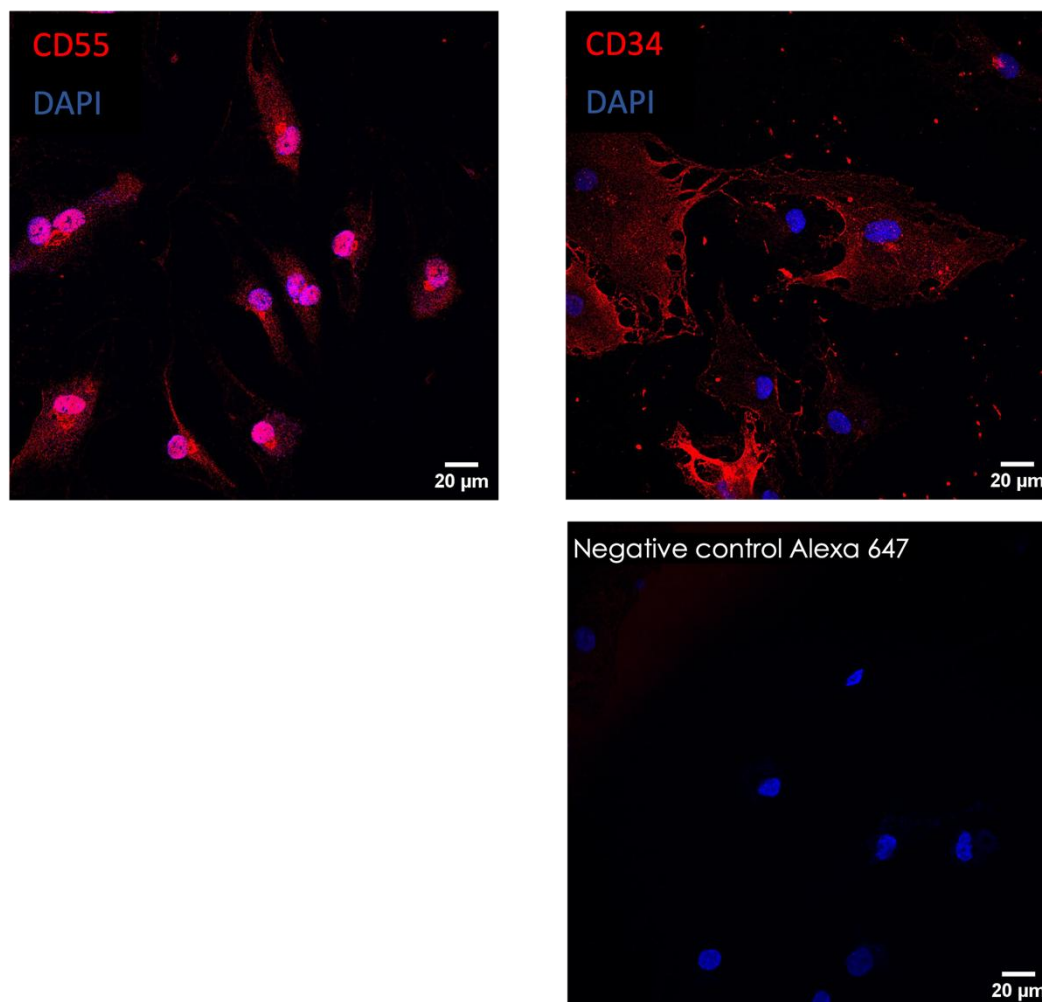


Figure 42. Expression of CD55 and CD34 in OA FLS by IF. Immunofluorescence confirming CD55 and/or CD34 expression in OA FLS after 3 days of tunicamycin stimulation (1 $\mu\text{g}/\text{mL}$). Nuclei were counterstained with DAPI. Negative control confirms the absence of nonspecific signal. FLS: fibroblast-like synoviocytes; IF: immunofluorescence.

6.3.2. ER stress proteins expression and secretion in FLS under ER stress

As an initial validation step, we first assessed the modulation of chaperone expression under ER stress. To this end, cells were treated with tunicamycin for 1, 3, or 7 days. This experiment showed significant upregulation of BiP, HYOU1, MANF, PDIA4, HSP90B1, LMAN1, and

CALR, whereas GANAB, TXNDC5, DNAJB11, and ERP29 remained unchanged. Except for BiP, most of these proteins were detectable at baseline (Figure 43).

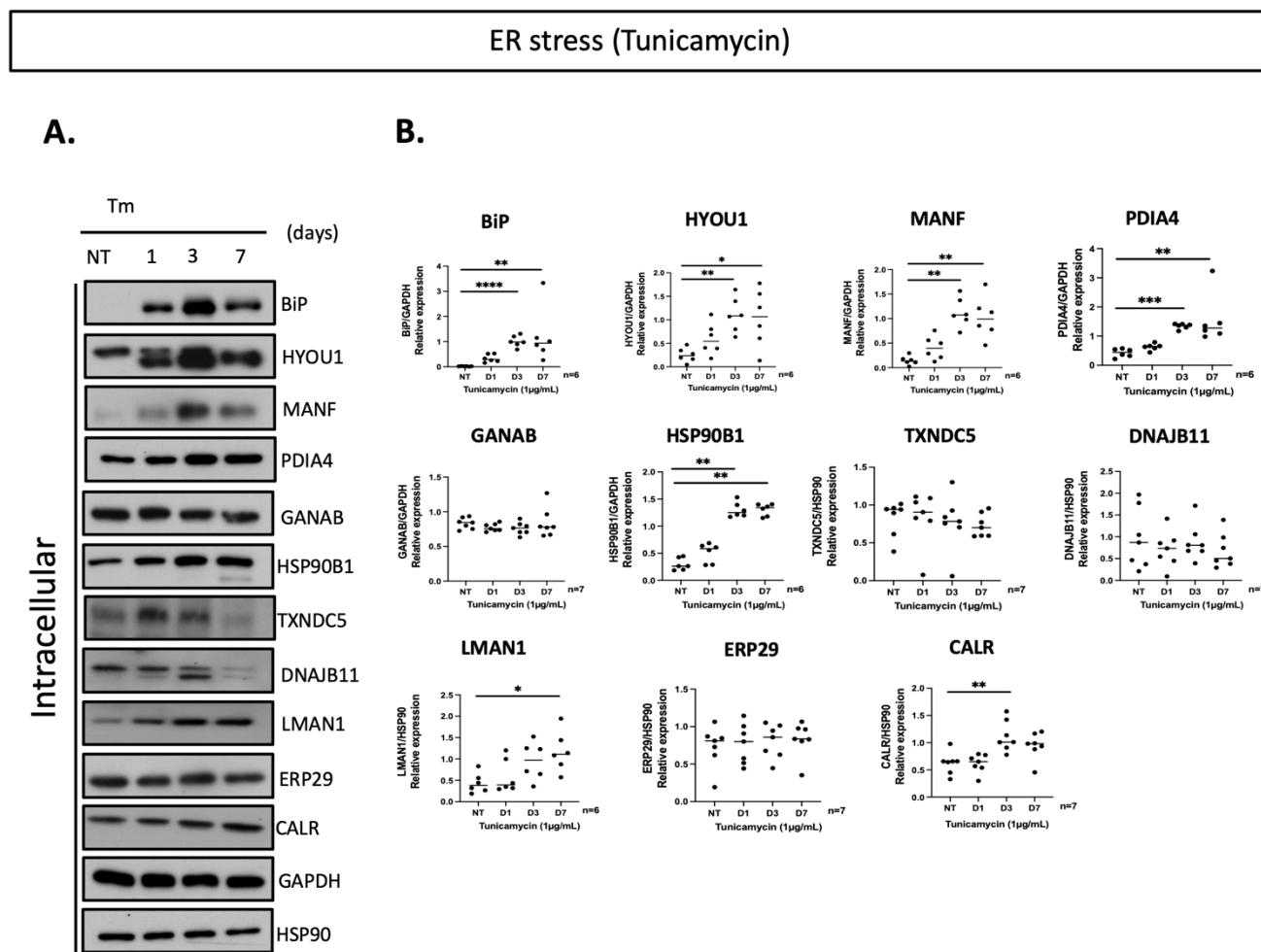


Figure 43. Modulation of ER stress proteins following induction of ER stress. A) Representative WB images showing intracellular expression of 11 ER stress proteins in FLS after 1, 3, and 7 days of stimulation with tunicamycin (Tm, 1 µg/mL), with B) their corresponding semi-quantifications. HSP90 or GAPDH served as loading controls. Statistical analysis was performed using the nonparametric paired Friedman test followed by Dunn's post hoc test for multiple comparisons. $n \geq 6$. NT: non-treated; D: day. Data are expressed as mean values. n: number of patients, NT: non-tretated; D:day; ER: endoplasmic reticulum; Tm: tunicamycin.

Given that ER chaperone proteins can themselves exert pro-inflammatory effects when secreted or exposed at the cell surface (Section 3.7), we next examined their secretion in parallel with the intracellular analysis. Prolonged tunicamycin exposure induced the release of BiP, PDIA4, HSP90B1, ERP29, and CALR, while PDIA4 was the only protein detectable in supernatants under unstimulated conditions. ERP29 secretion was observed exclusively at day 7 (Figure 44). HYOU1, MANF, GANAB, TXNDC5, DNAJB11, and LMAN1 were not secreted under any condition tested (data not shown).

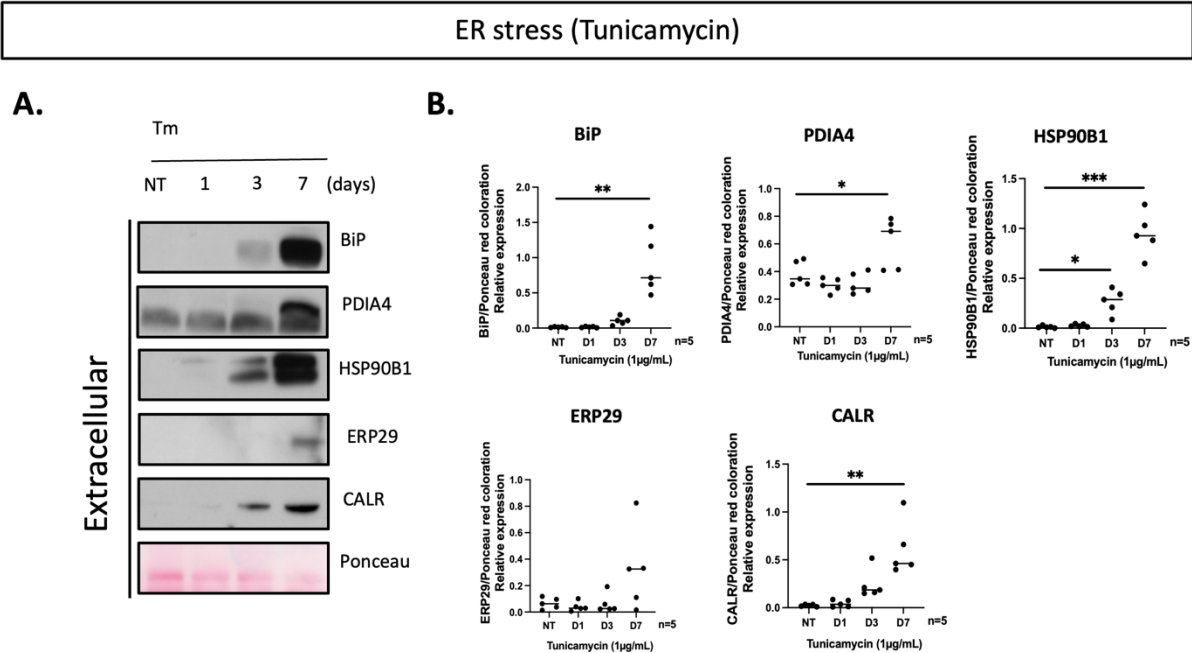


Figure 44. Secretion of ER stress proteins following induction of ER stress. A) Representative WB of matched supernatants after stimulation of FLS with 1, 3 or 7 days of tunicamycin. Ponceau S staining used as loading control and B) their semi-quantification. Statistical analysis was performed using the nonparametric paired Friedman test followed by Dunn’s post hoc test for multiple comparisons. n=5. n: number of patients; NT: non-treated; D: day; ER: endoplasmic reticulum; Tm: tunicamycin. Data are expressed as mean values.

6.3.3. ER stress proteins expression in FLS under inflammatory conditions

To mimic the inflammatory microenvironment characteristic of synovitis and address the self-perpetuating cycle between inflammation and ER stress described in Section 3.7, we next investigated how pro-inflammatory stimulation with TNF- α modulates ER chaperone protein expression in FLS. Under inflammatory stimulation (TNF- α), BiP increased from day 1, while HYOU1, MANF, and PDIA4 rose from day 3 (Figure 45).

Inflammation (TNF- α)

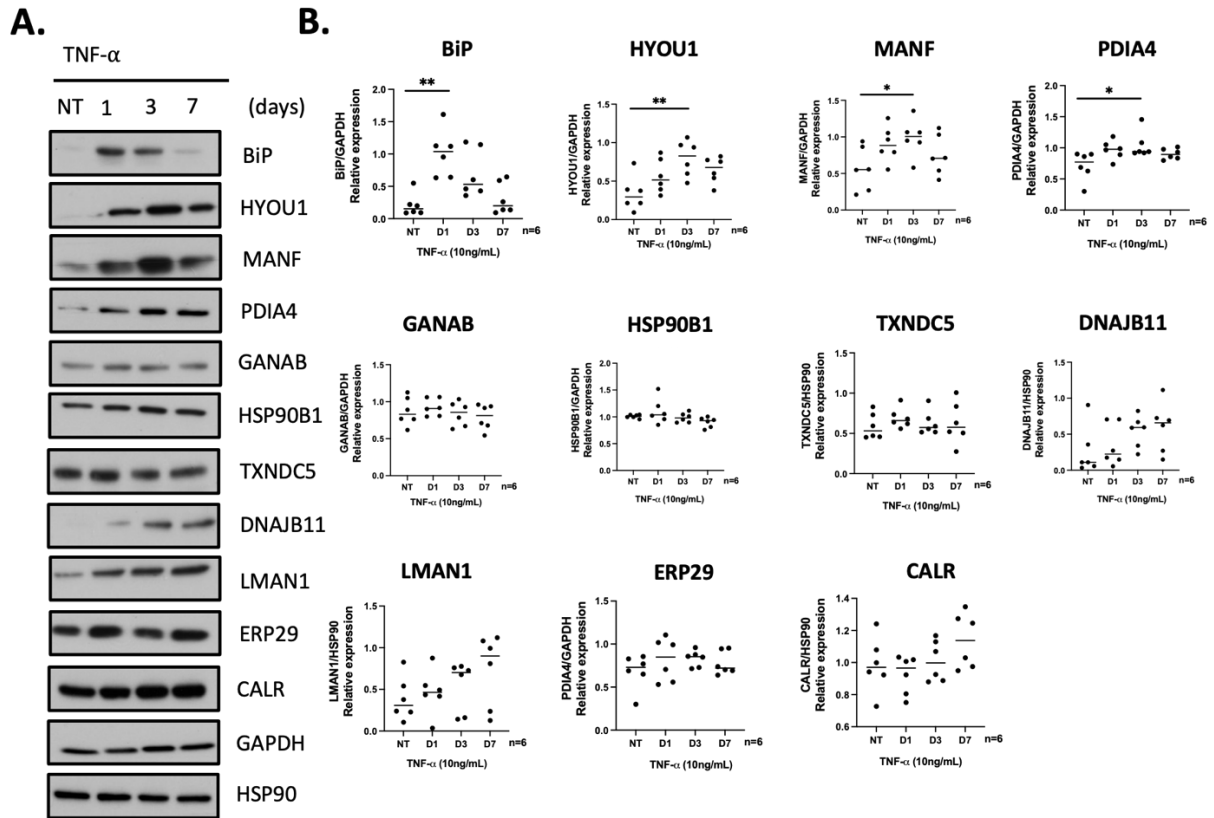
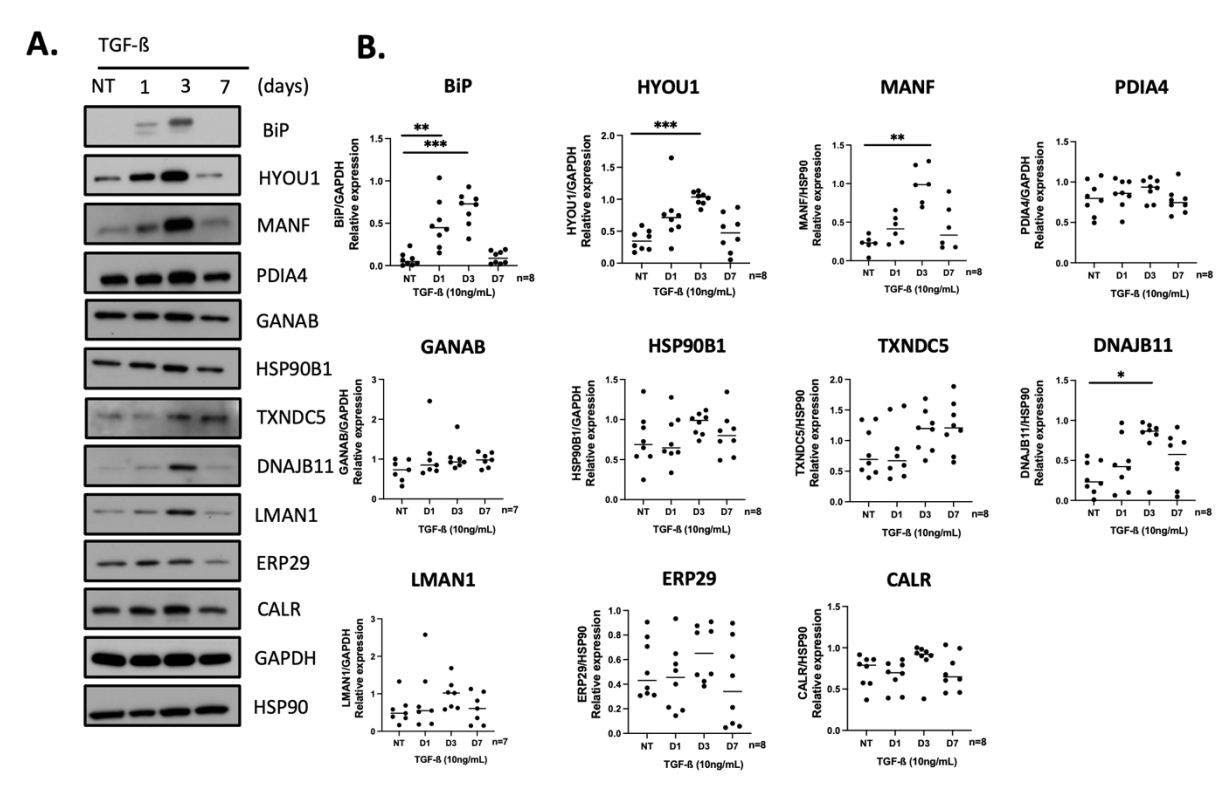


Figure 45. Modulation of ER stress proteins following induction of inflammation. A) Representative WB images showing intracellular expression of 11 ER stress proteins in FLS after 1, 3, and 7 days of stimulation with TNF- α (10 ng/mL) with B) their corresponding semi-quantifications. HSP90 or GAPDH served as loading controls. Statistical analysis was performed using the nonparametric paired Friedman test followed by Dunn's post hoc test for multiple comparisons. n=6. NT: non-treated; D: day. Data are expressed as mean values. n: number of patients, NT: non-treated; D: day. ER: endoplasmic reticulum; TNF- α : tumor necrosis factor- α .

6.3.4. ER stress protein expression in FLS following TGF- β stimulation

Following inflammatory stimulation, we next assessed whether TGF- β stimulation, a known driver of fibrotic responses, modulates ER chaperone protein expression in FLS. This stimulation increased BiP expression at days 1 and 3, and increased HYOU1, MANF, and DNAJB11 expression at day 3 (Figure 46).

Fibrotic process (TGF-β)



- ✓ OA FLS express both CD55 and CD34 at baseline; FACS sorting necessary
- ✓ ER stress induces upregulation of BiP, HYOU1, MANF, PDIA4, HSP90B1, LMAN1 and CALR, and secretion of BiP, PDIA4, HSP90B1, ERP29 and CALR.
- ✓ Inflammatory stimulation (TNF- α) increases BiP, HYOU1, MANF and PDIA4.
- ✓ Pro-fibrotic stimulation (TGF- β) increases BiP, HYOU1, MANF and DNAJB11.

6.4. Selection of key ER stress proteins associated with fibrosis-related synovitis

The combined findings of the IHC analyses, the CIS mouse model, and the *in vitro* experiments revealed that HYOU1, MANF, PDIA4, DNAJB11, and CALR are more prominently modulated in inflammatory and pro-fibrotic contexts (Table 11). However, their involvement in synovitis has not yet been well characterized in the literature. Therefore, these proteins served as the basis for our subsequent functional investigations.

	IHC		WB			
	Cohort 1	CIS (mouse)	Primary FLS			
			ER stress (Tm)		Fibrosis (TGF- β)	Inflammation (TNF- α)
	Tissue	Tissue	Intracellular	Extracellular	Intracellular	Intracellular
BiP			X	X	X	X
HYOU1			X		X	X
MANF	X	X	X		X	X
PDIA4	X	X	X	X		X
GANAB	X					
HSP90B1		X	X	X		
TXNDC5	X					
DNAJB11		X			X	
LMAN1		X	X			
ERP29				X		
CALR	X	X	X	X		

Table 11. Summary table of ER stress proteins found to be overexpressed across all experiments. Each cross indicates the overexpression or secretion of the protein of interest under pathological conditions (ER stress, pro-fibrotic, or pro-inflammatory processes), as determined by IHC or WB analysis. ER: endoplasmic reticulum; IHC: immunohistochemistry; WB: western blotting.

6.5. UPR pathway–dependent regulation of ER stress proteins in FLS

We next sought to identify which UPR pathways regulate the expression of HYOU1, MANF, PDIA4, DNAJB11, and CALR in OA FLS. To address this, cells were pretreated for 4 hours with selective inhibitors of the three main UPR branches, IRE1 α (4 μ 8c), ATF6 (Ceapin-A7) or PERK (PERK-IN-2), prior to a 24-hour stimulation with tunicamycin.

To assess IRE1 α pathway modulation, phosphorylation of IRE1 α (p-IRE1 α) was monitored as a readout of pathway inhibition. Under these conditions, HYOU1 expression was altered, suggesting that this chaperone may be regulated downstream of the IRE1 α branch of the UPR (Figure 47).

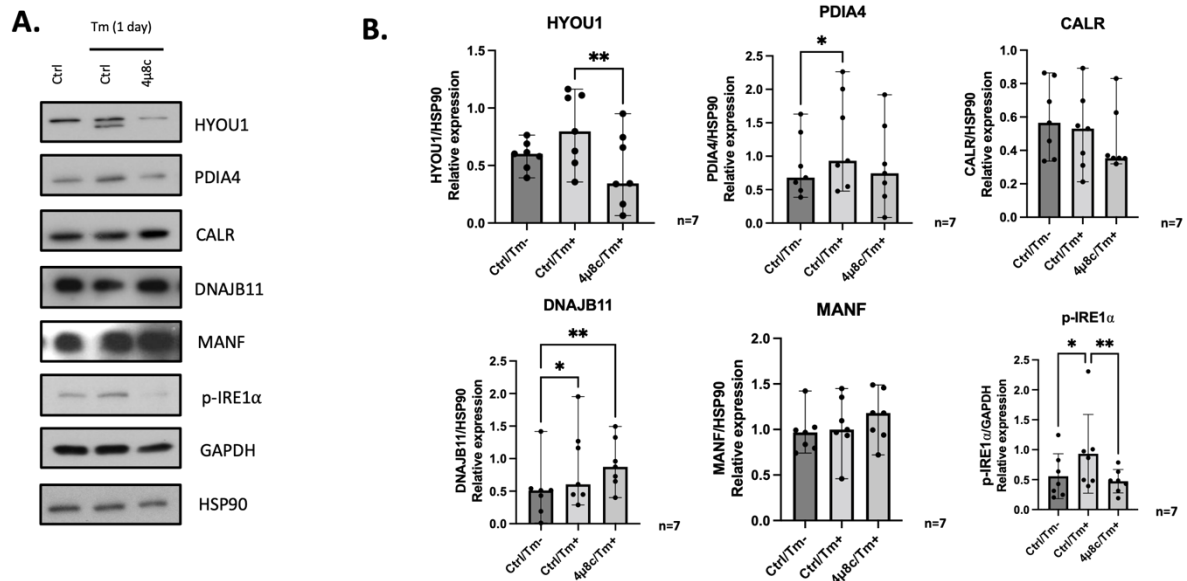


Figure 47. Modulation of IRE1 α signaling and downstream ER stress proteins following ER stress induction and pharmacological inhibition of IRE1 α . A) Representative WB images showing intracellular expression of ER stress proteins, including phosphorylated IRE1 α (p-IRE1 α), in cells stimulated with tunicamycin in the presence or absence of the IRE1 α inhibitor 4 μ 8c (4h prior), and their B) corresponding semi-quantifications of the indicated proteins. HSP90 or GAPDH served as loading controls. Statistical analysis was performed using the nonparametric paired Friedman test followed by Dunn's post hoc test for multiple comparisons. n=7. Data are expressed as median values. Ctrl: control; Tm: tunicamycin; ER: endoplasmic reticulum.

Effective inhibition of the ATF6 pathway by Ceapin-A7 was supported by a decrease in the cleaved ATF6 form (~50 kDa), while the full-length ATF6 (~90 kDa) remained unchanged. Consistently, the expression of HYOU1 and PDIA4 was altered, suggesting that these genes are modulated downstream of the ATF6 branch of the UPR (Figure 48).

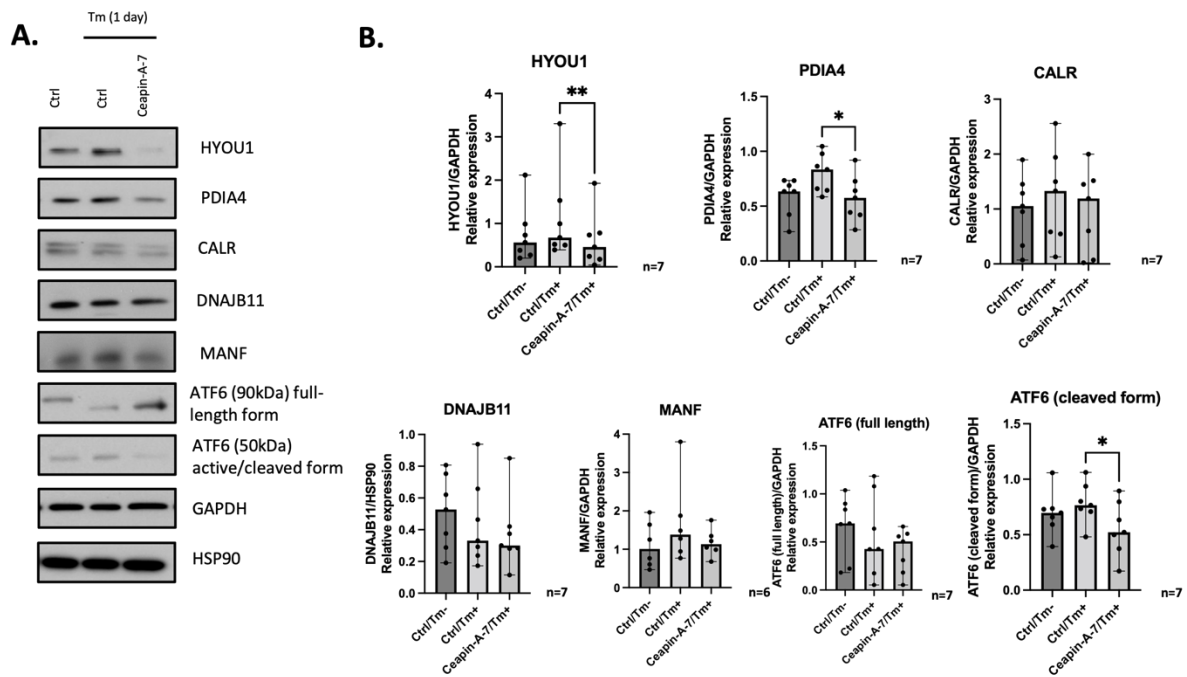


Figure 48. Modulation of ATF6 signaling and downstream ER stress proteins following an ER stress and pharmacological inhibition of ATF6. A) Representative WB images showing intracellular expression of ER stress-related proteins, including full-length ATF6 (~90 kDa) and cleaved ATF6 (~50 kDa), in cells stimulated with tunicamycin in the presence or absence of the ATF6 inhibitor Ceapin-A7 and their B) corresponding semi-quantifications of the indicated proteins. HSP90 or GAPDH served as loading controls. Statistical analysis was performed using the nonparametric paired Friedman test followed by Dunn's post hoc test for multiple comparisons. n=7. Data are expressed as median values. Ctrl: control; Tm: tunicamycin; ER: endoplasmic reticulum; UPR: unfolded protein response.

The effective modulation of the PERK branch was confirmed by a decrease in EIF2 α phosphorylation. However, in OA FLS, inhibition of the PERK pathway did not lead to detectable changes in the expression of ER stress proteins (Figure 49).

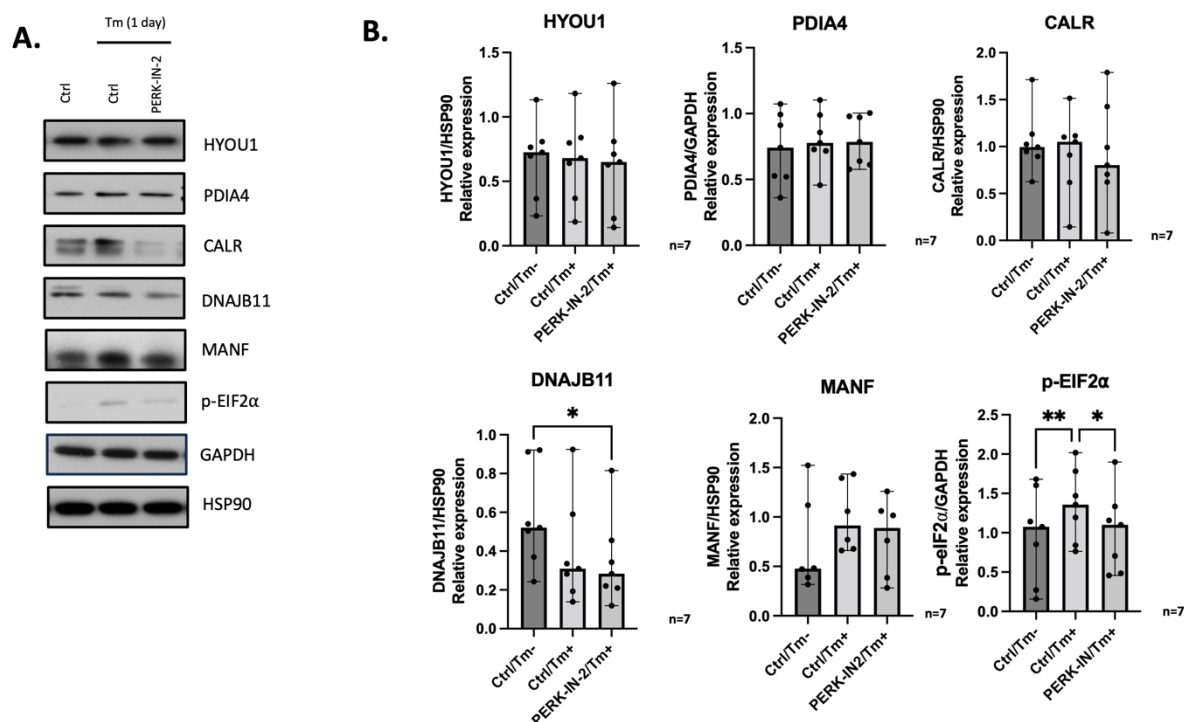


Figure 49. Modulation of the PERK branch of the UPR in OA FLS. A) Representative WB images showing intracellular expression of ER stress proteins in OA FLS stimulated 24h with tunicamycin with a prior (or not) 4h inhibition with PERK-IN-2 and their B) corresponding semi-quantifications of the indicated proteins. HSP90 or GAPDH served as loading controls. Statistical analysis was performed using the nonparametric paired Friedman test followed by Dunn's post hoc test for multiple comparisons. n=7. Data are expressed as median values. NT: non-treated; Tm: tunicamycin; ER: endoplasmic reticulum; UPR: unfolded protein response.

✓ **HYOU1 expression is modulated by the IRE1α and ATF6 pathways whereas PDIA4 only by ATF6 pathway in OA FLS.**

6.6. Role of PDIA4 in TGF-β–driven pathogenic mechanisms in osteoarthritic synovitis

6.6.1. HYOU1, MANF, PDIA4, DNAJB11 and CALR do not modulate TGF-β–induced responses in OA FLS

Having identified ER stress proteins modulated under TGF-β and pro-inflammatory stimulations, we next addressed whether ER stress proteins themselves modulate TGF-β– and inflammation-induced responses in FLS.

Fibrosis represents one of the three key hallmarks of synovitis, alongside inflammation and hyperplasia, and was therefore investigated first. We specifically examined whether HYOU1, MANF, PDIA4, DNAJB11, or CALR influence fibrotic responses driven by fibroblast-to-myofibroblast transition (FMT), a central mechanism of synovial fibrosis characterized by α-SMA expression in myofibroblasts upon TGF-β stimulation [285].

To determine whether silencing of ER stress proteins (Figure 50A–B) directly affects FMT, FLS were transfected with siCtrl, siHYOU1, siDNAJB11, siPDIA4, siCALR, or siMANF, and α -SMA and fibronectin expression were assessed. As expected, TGF- β stimulation for 3 days induced FMT, as evidenced by the upregulation of α -SMA and fibronectin expression. However, no significant modulation of FMT was observed following silencing of these ER stress proteins under TGF- β stimulation (Figure 50C).

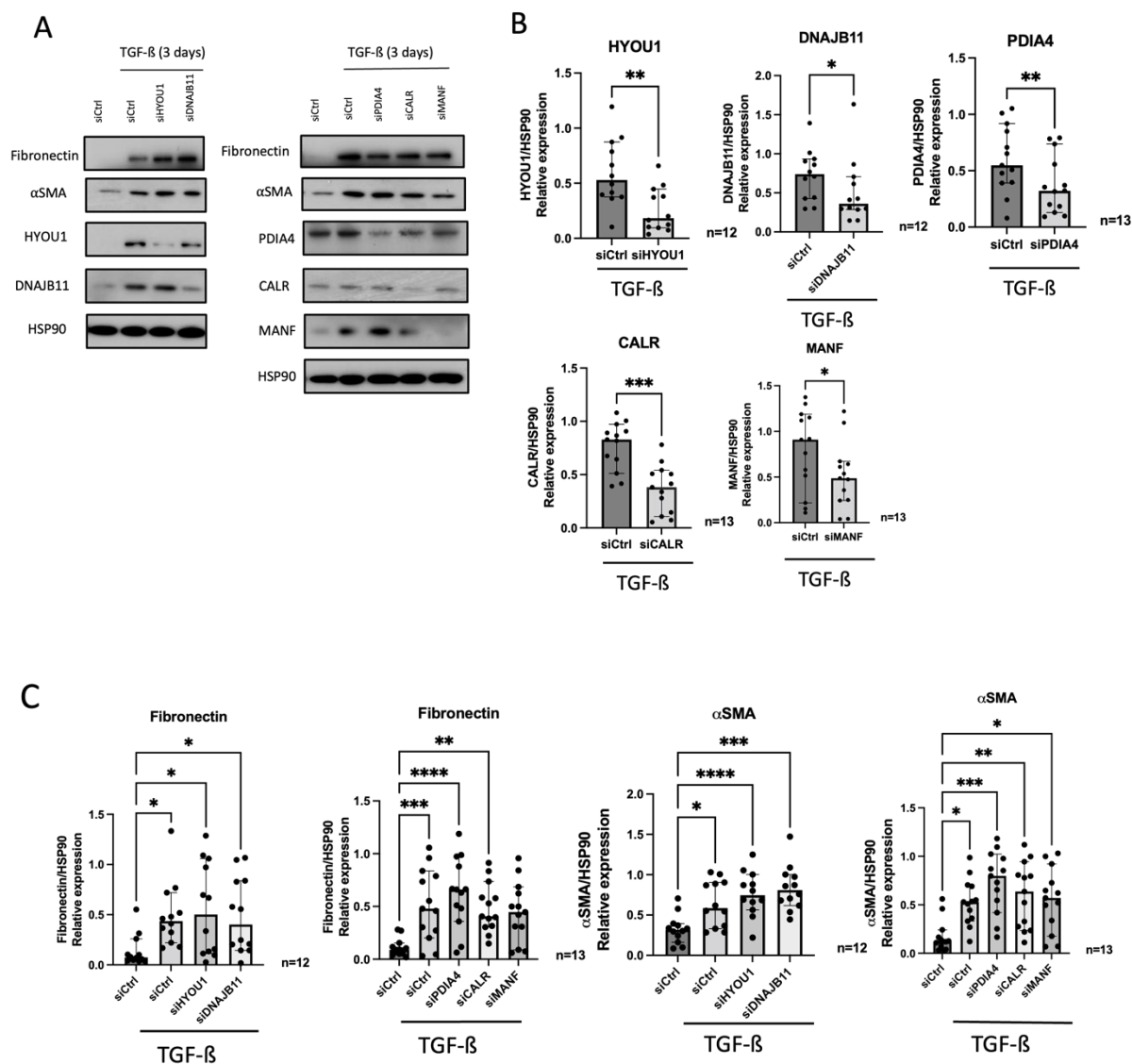


Figure 50. Effect of HYOU1, DNAJB11, PDIA4, CALR or MANF silencing on FMT in FLS. A) Representative WB images showing intracellular expression of fibronectin, α -SMA, HYOU1, DNAJB11, PDIA4, CALR and MANF following 24-hours pretreatment with their respective siRNA and subsequent 3-day stimulation with TGF- β , along with B) and C) their corresponding semi-quantifications. Statistical analysis was performed using the nonparametric paired Friedman test followed by Dunn's post hoc test for multiple comparisons. Each point represents an individual donor, and results are presented as the median with a 95% confidence interval. FMT: fibroblast–mesenchymal transition FLS: fibroblast-like synoviocytes; WB: western blotting.

6.6.2. HYOU1, MANF, PDIA4, DNAJB11 and CALR are not involved in pro-inflammatory processes in OA FLS

In the same experimental approach, 3-day TNF- α stimulation induced a marked inflammatory response, reflected by elevated IL-6 secretion. Despite efficient knockdown of each ER stress protein (Figure 51A–B), no significant change in TNF- α –induced IL-6 levels was detected (Figure 51C).

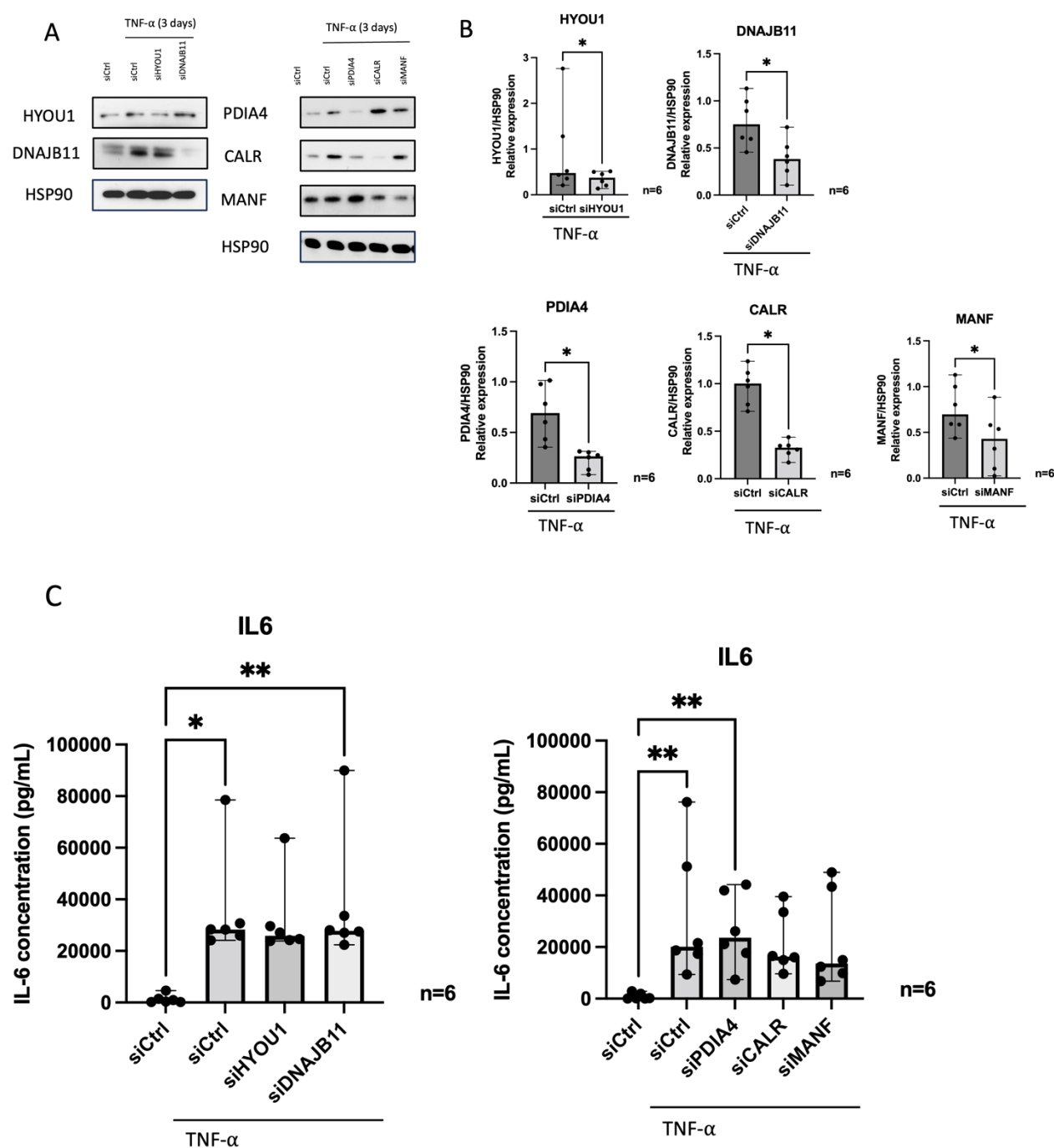


Figure 51. Effect of HYOU1, DNAJB11, PDIA4, CALR or MANF silencing on inflammation in FLS. A) Representative WB images showing intracellular expression of HYOU1, DNAJB11, PDIA4, CALR and MANF with B) their corresponding semi-quantifications and C) measurement of IL-6

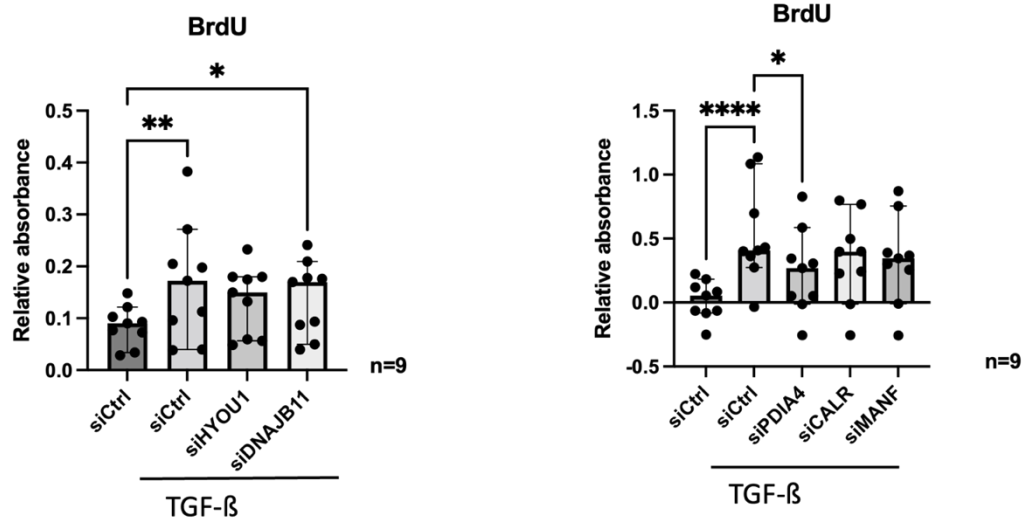
(inflammation) secretion using ELISA, following 24-hours pretreatment with their respective siRNA and subsequent 3-day stimulation with TNF- α . Each point represents an individual donor, and results are presented as the median with a 95% confidence interval. Statistical analysis was performed using the nonparametric paired Friedman test followed by Dunn's post hoc test for multiple comparisons. FLS: fibroblast-like synoviocytes; WB: western blotting.

Our findings indicate that, in FLS, silencing HYOU1, DNAJB11, PDIA4, CALR, or MANF did not significantly alter fibrotic or inflammatory responses under the conditions tested. These results suggest that, within FLS, the contribution of these ER stress proteins to synovitis may involve alternative mechanisms, potentially linked to another hallmark of synovitis such as fibroblast proliferation (hyperplasia). However, they do not exclude additional roles in other synovial cell populations (macrophages).

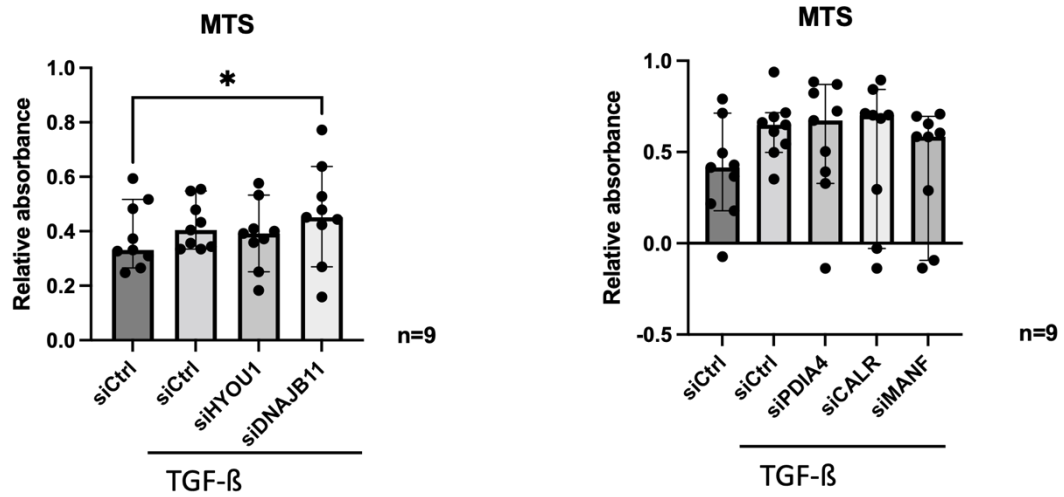
6.6.3. PDIA4 promotes FLS proliferation via the TGF- β pathway

Because fibroblasts drive synovial hyperplasia, we tested whether the selected five ER stress proteins contribute to this proliferative process. To address this, FLS were pre-transfected with siRNAs targeting each protein 24 hours before exposure to TGF- β , a potent pro-fibrotic stimulus, for three days. As expected, TGF- β induced a marked proliferative response in FLS (siCtrl without TGF- β vs. siCtrl + TGF- β), which was significantly regulated by PDIA4 (Figure 52A). Silencing HYOU1, PDIA4, DNAJB11, MANF, or CALR did not affect cell viability (Figure 52B), and knockdown efficiency was verified by WB (Figure 52C and 52D for qualitative and quantitative analysis, respectively).

A



B



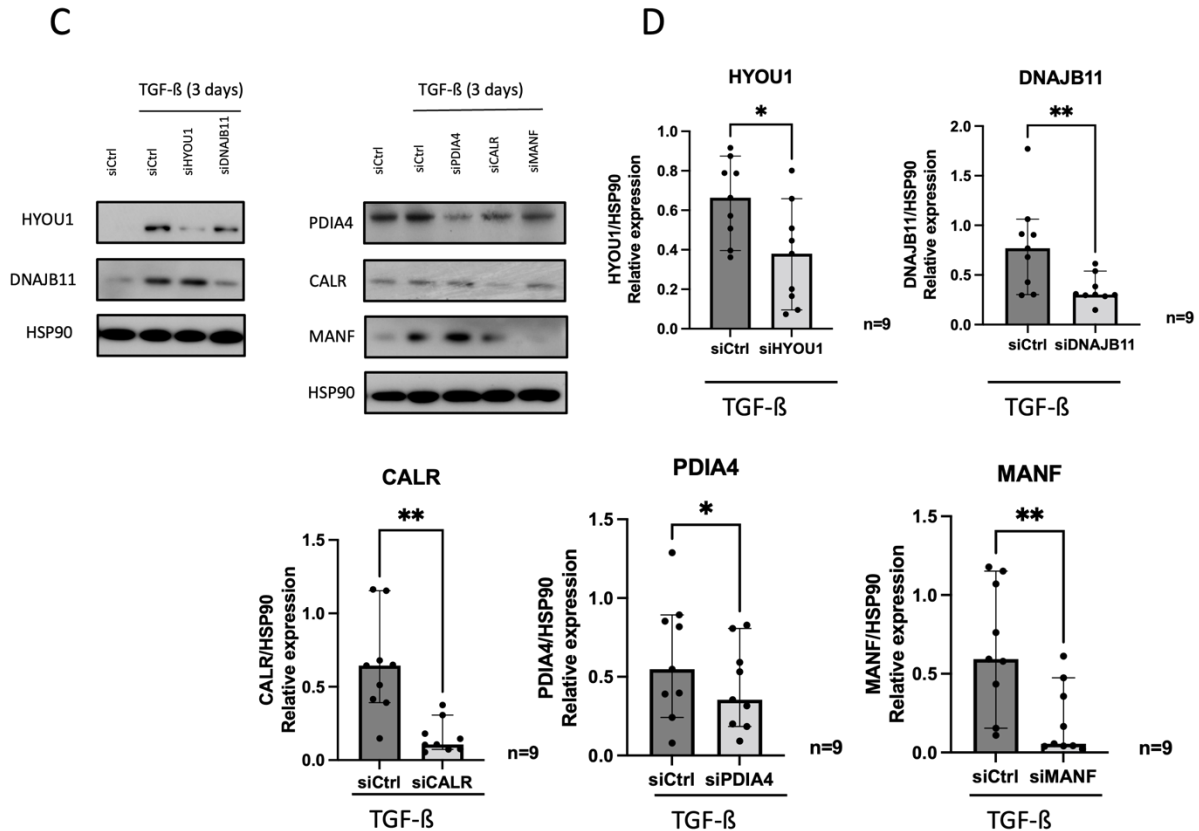


Figure 52. Effect of ER stress protein silencing on FLS proliferation. FLS were transfected with siRNAs targeting HYOU1, MANF, PDIA4, DNAJB11, or CALR, then stimulated with TGF- β (10 ng/mL) for 3 days. A) Proliferation was measured by BrdU incorporation, and B) viability by MTS assay, showing no impact of silencing on cell survival. Efficient knockdown of each target protein was assessed with the C) their semi-quantification by WB. Data are presented as median with 95% CI; each point represents one patient. Statistical significance was assessed using the Wilcoxon paired test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). n: number of patients, Ctrl: controls; D:day. ER: endoplasmic reticulum; WB: western blotting; TGF- β : transforming-growth factor- β ; siRNA: small interfering. BrdU: bromodesoxyuridine.

- ✓ Depletion of HYOU1, MANF, PDIA4, DNAJB11 and CALR does not significantly affect fibroblast-to-myfibroblast transition (α -SMA, fibronectin) under TGF- β stimulation.
- ✓ Silencing of these ER stress proteins does not modify TNF- α -induced IL-6 secretion in OA FLS under tested conditions.
- ✓ siPDIA4 significantly reduces TGF- β -induced OA FLS proliferation without affecting viability

6.7. Summary of the results

1. The ER stress proteins previously identified by mass spectrometry as correlating with the inflammatory continuum based on the Tak score across synovial biopsies from patients with OA, CPPD, and RA could be localized by IHC within the same synovial membranes (Cohort 1). They were restricted to the lining layer in NIN samples (Tak < 4) and progressively

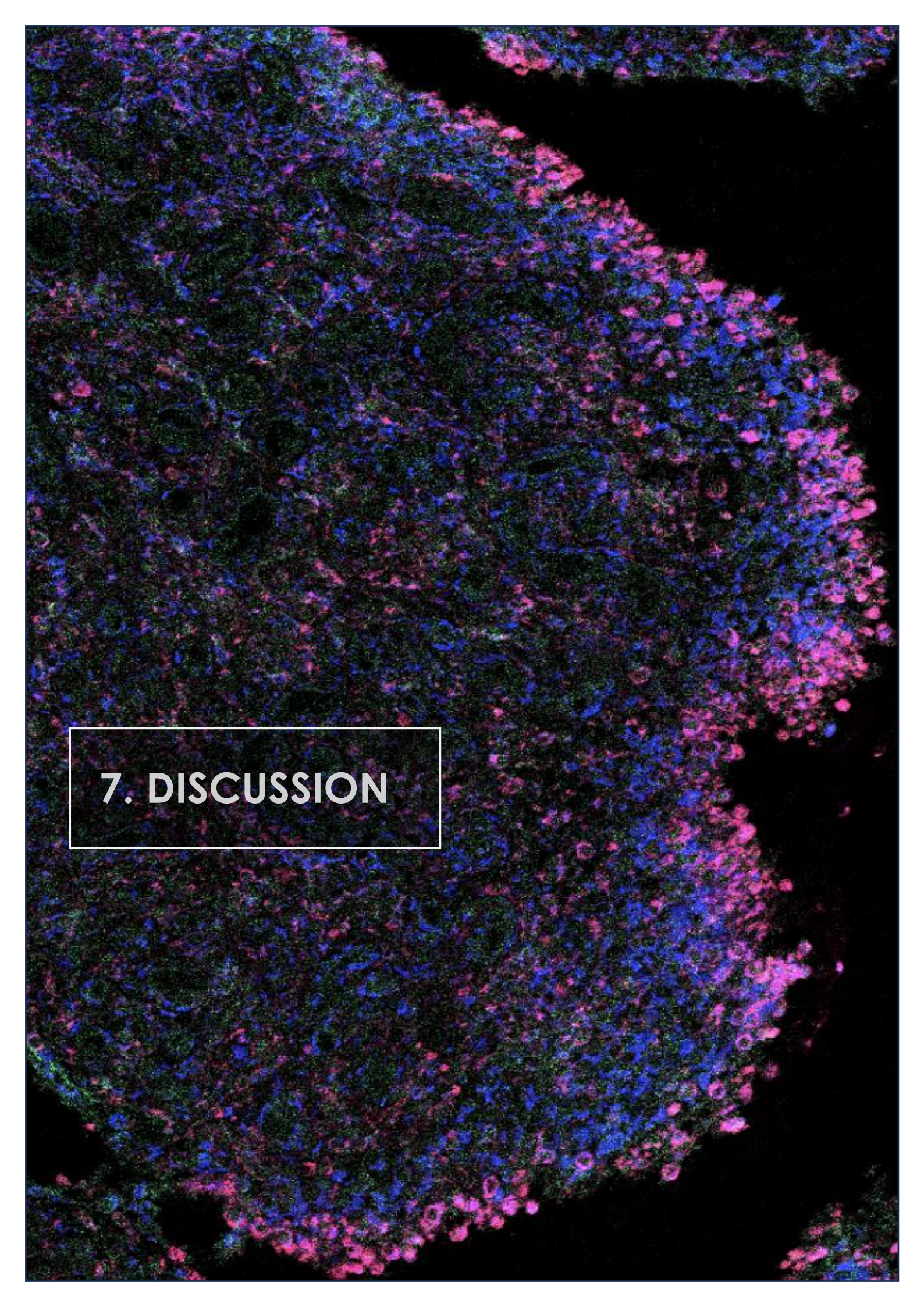
infiltrated the sublining layer as inflammation increased ($Tak \geq 4$). These results were confirmed on Cohort 2 by IMC.

2. The CIS model overcame the lack of controls in the human samples and confirmed the findings described in point 1. Moreover, MANF, PDIA4, HSP90B1, DNAJB11, LMAN1, and CALR were overexpressed in the CIS model compared to controls. MANF, LMAN1, and DNAJB11 showed a positive correlation with pro-inflammatory cytokines (IL1 β and/or IL6); PDIA4, HSP90B1, and CALR with the fibrotic marker *Acta2*; and MANF, HSP90B1, DNAJB11, LMAN1, and CALR with synovial thickness.

3. Imaging mass cytometry (Cohort 2) and further confirmation by IF on an extended cohort (Cohort 3) revealed that these ER stress proteins are expressed by FLS. In NIN OA, where the proteins are restricted to the lining layer, they co-localize with CD55⁺ FLS. As inflammation develops and the proteins are overexpressed in the sublining, they co-express with FLS exhibiting a CD34⁺ phenotype. FLS expressing ER stress proteins may undergo a transitional state characterized by co-expression of CD55 and CD34.

4. *In vitro*, induction of ER stress in FLS (expressing both CD55 and CD34) led to the overexpression of BiP, HYOU1, MANF, PDIA4, HSP90B1, LMAN1, and CALR, along with the secretion of BiP, PDIA4, HSP90B1, ERP29, and CALR. Under pro-inflammatory conditions, BiP, HYOU1, MANF, and PDIA4 were modulated, while under TGF- β stimulation, BiP, HYOU1, and MANF were overexpressed. Pharmacological inhibition of the UPR pathways indicated that HYOU1 and PDIA4 are regulated downstream of the ATF6 branch, whereas HYOU1 also appears to be modulated by the IRE1 α pathway. Functional assays suggest that the role of PDIA4 in synovitis can mediate FLS proliferation but not induce myofibroblast differentiation or the IL-6 inflammatory pathway, either as the other investigated chaperones.

The results presented in this thesis are currently under peer review for publication.



7. DISCUSSION

7.1. Localization of ER stress proteins in the human inflamed synovial membranes and within cell subpopulations

The previous proteomic analysis, performed in the laboratory in 2020, identified eleven ER stress proteins, including BiP, HYOU1, MANF, PDIA4, GANAB, HSP90B1, TXNDC5, DNAJB11, LMAN1, ERP29 and CALR, as correlating with the inflammatory Tak score across OA, CPPD, and RA synovial tissues [13]. These findings, together with growing evidence that ER stress is emerging as a key driver of synovitis, amplifying inflammatory signaling [268], promoting fibroblast proliferation [263], angiogenesis [286], and fibrotic remodeling [267], prompted us to investigate in the present study the histological distribution of ER stress proteins within inflamed human OA synovium.

Consistent with our observations, previous studies have localized some of these proteins in synovial tissue: BiP, HSP90B1, TXNDC5 and CALR by IHC and/or IF in both OA and RA [263,287–289], and MANF in RA [290], with staining detected in the lining and sublining layers. Importantly, both MANF, TXNDC5 and CALR were reported to be overexpressed in RA tissues, in line with our findings and reinforcing their contribution to synovial pathology [288–290]. However, unlike our study, these works did not capture the dynamic distribution pattern that we observed, where proteins were predominantly confined to the lining in mildly inflamed synovium (Tak score < 4) but progressively expanded into the sublining with increasing inflammation (Tak score $\geq$ 4). To validate these observations, IMC was performed on a second independent cohort of OA and RA samples. This approach not only confirmed the progressive overexpression of ER stress proteins in the sublining but also revealed the spatial dynamics of synovial cells. In NIN OA, CD55⁺ FLS were largely confined to the lining, while inflammation (in IN OA and RA) was associated with the expansion of CD34⁺CD31⁻ FLS in the sublining. The distribution pattern of CD68⁺ MLS was similar to that of CD55⁺ FLS in OA. However, CD68⁺ MLS unlike CD55⁺ FLS remained detectable throughout RA tissue. These findings are consistent with the description by Culemann *et al.* [162] of an MLS-formed palisade-like structure that overlays and lines the RA FLS. This structure forms a tight junction barrier similar to that found in other epithelial layers. While this barrier helps to limit synovial inflammation, it becomes disrupted as inflammation progresses. This leads to the loss of MLS contacts and allows further infiltration of the FLS into the synovium. These observations are also in line with Mucke *et al.* study that identified by IHC a predominance of CD55⁺ FLS and CD68⁺ MLS in the lining of OA synovium, although without analyzing their distribution according to inflammatory gradients, as reported in our study [291].

7.2. Validation of ER stress proteins overexpression in Collagenase-Induced Synovitis Mouse Model

To address the scarcity of healthy human synovial tissue, the CIS mouse model was implemented. This model induces synovitis in mice through intra-articular collagenase injections. Synovial inflammation of the CIS mouse model was confirmed by the increase in synovial thickness due to hyperplasia but also by the expression of the pro-inflammatory cytokines (IL-1 β and IL-6 mRNA levels) [200]. PDIA4, HSP90B1 and CALR were expressed even in controls but with a higher expression level when the synovium was inflamed. The expression levels of BiP did not increase in CIS models, probably because of its involvement in an earlier phase of ER stress and inflammation [292]. The expression levels of MANF, PDIA4, HSP90B1, DNAJB11, LMAN1 and CALR were significantly overexpressed in inflamed CIS synovium compared to non-inflamed synovium [with a positive correlation vs synovial thickness for all of them (except BiP and PDIA4)] reinforcing the potential role of the ER stress proteins in synovitis. Interestingly, PDIA4, HSP90B1 and CALR correlated with *Acta2* mRNA levels, suggesting their involvement in the pro-fibrotic processes. It is of particular interest since previous work in human RA synovium demonstrated that HSP90B1 expression correlated with synovial thickness. However, in contrast to our findings, HSP90B1 expression was shown to correlate with inflammatory parameters but not with fibrotic markers [287]. MANF and LMAN1 correlated with IL-6 and IL-1 β mRNA expression, strengthening the link between ER stress protein expression and inflammation.

7.3. Coexpression of ER stress proteins with FLS subtypes

We had previously shown that FLS subsets and ER stress proteins displayed similar spatial patterns during synovial inflammation. Using IMC, we have demonstrated that these proteins are expressed by specific FLS subsets. In NIN OA, they were mainly associated with CD55⁺ FLS within the lining, whereas in IN OA their expression shifted toward CD34⁺ FLS in the sublining. CD68⁺ MLS also expressed these proteins, mainly in the lining under NIN conditions but in a lesser proportion than CD55⁺ FLS. This distribution was validated by IF in an extended cohort of 25 patients, using PDIA4 as a reference marker. In this analysis, PDIA4 was initially detected in CD55⁺ FLS and, to a lesser extent, CD68⁺ MLS in the lining of NIN OA, but its presence decreased in the lining with inflammation. Meanwhile, the proportion of PDIA4⁺CD34⁺CD31⁻ cells significantly increased in the sublining. In situ reports describe TXNCD5 as primarily expressed in FLS, with strong expression in RA synovial membranes

and much weaker expression in OA, without distinguishing fibroblast subsets [289]. *In vitro*, the spliced form of XBP1, an ER stress protein, has been reported in RA FLS [269]. Our ER stress proteins of interest, including BiP [263], HYOU1 [293], MANF [290], TXNDC5 [294,295], and CALR [296] were also shown to be expressed in RA FLS. No co-expression with lymphocyte markers was detected. These findings emphasize the predominant role of FLS in ER stress protein expression.

7.4.ER stress protein expression and secretion by FLS under ER stress, pro-inflammatory, and TGF- β stimulations

Single-cell studies have demonstrated that RA synovitis is associated with marked heterogeneity among FLS populations [179,180], with CD55⁺ lining FLS and CD34⁺ sublining FLS displaying distinct phenotypes. Especially, CD55⁺ lining OA FLS are associated with a more homeostatic profile, whereas CD34⁺ sublining FLS show increased IL-6 expression [181] and upregulation of pro-fibrotic genes [183]. Interestingly, in mouse models, intermediate OA fibroblast states have been described, characterized by cells co-expressing lining (Prg4) and myofibroblast/sublining (Tagln) markers, suggesting transitional states between lining and sublining populations [209]. Of note, several human studies have highlighted the contribution of some key ER stress proteins to the inflammatory reprogramming of FLS. The spliced form of XBP1 directly links ER stress to the production of inflammatory cytokines such as IL-6 and TNF- α in RA FLS [269], while knockdown of IRE1 was shown to impair the pathogenic transformation of these cells [265]. In addition, pharmacological induction of ER stress with thapsigargin markedly increased IL-6 and IL-8 expression in RA FLS [297]. In line with this, our data suggest that PDIA4 may contribute to a phenotypic transition, as indicated by the negative correlation between CD55⁺PDIA4⁺ FLS in the lining and CD34⁺ PDIA4⁺ FLS in the sublining, a pattern not observed in FLS lacking PDIA4 expression. Accordingly, we next investigated, *in vitro*, the level of expression of ER stress chaperones in FLS, hypothesising that they differ between NIN FLS and IN FLS. Although the expression of CD55 and/or CD34 in FLS was validated by IF and/or Western blot in our *in vitro* culture system, this approach did not allow determination of the relative proportions of the four FLS phenotypes (CD55⁺/CD34⁻, CD55⁺/CD34⁺, CD55⁻/CD34⁺ CD55⁻/CD34⁻). This limitation is primarily due to technical constraints inherent to IF, as simultaneous co-localization of CD55, CD34, and PDIA4 could not be achieved because of antibody species incompatibilities. Consequently, we were unable to monitor phenotype transitions under pro-inflammatory or pro-fibrotic conditions, nor to assess changes in PDIA4 expression across these distinct FLS subsets or following PDIA4

silencing. As a result, we could not directly address whether PDIA4 contributes to the phenotypic transition of FLS from a CD55⁺ lining-like state toward a CD34⁺ sublining-like phenotype. Future studies combining flow cytometry-based cell sorting with intracellular PDIA4 detection would be well suited to overcome these limitations. Such an approach would allow isolation of defined FLS subsets based on CD55 and CD34 expression and enable quantitative assessment of PDIA4 levels across inflammatory states, thereby providing deeper insight into the potential role of PDIA4 in FLS phenotypic plasticity. All chaperone proteins investigated, excluding BiP, were expressed in unstimulated FLS, suggesting a role in maintaining basal cellular homeostasis. In contrast, their expression was markedly increased under ER stress, pro-inflammatory, and/or TGF- β stimulations. In our model, BiP, HYOU1, and MANF were consistently upregulated across all three stimulatory contexts. PDIA4 and CALR appeared particularly relevant, as they were not only overexpressed but also secreted by FLS under prolonged ER stress. Additionally, PDIA4 expression was enhanced in pro-inflammatory conditions, while DNAJB11 showed a specific increase under TGF- β stimulation. Beyond their intracellular upregulation, several chaperones can also be secreted, in autoimmune contexts such as RA, and once released, they may acquire pro-inflammatory properties by stimulating cytokine production [273,276]. In line with previous reports, beyond their intracellular roles, ER chaperones can be released extracellularly and exert cell type-specific functions: CALR activates macrophages in autoimmune diseases [273], ER stress-induced secretion of CALR [273], BiP [298] and HSP90B1 [276] from fibroblasts promote dendritic cell cytokine responses, while PDIA4 [299] and ERP29 [300] regulate platelet function and haemostasis. Based on these findings, we focused on HYOU1, MANF, PDIA4, DNAJB11, and CALR to assess their potential role in fibrosis, inflammation and proliferation of FLS.

7.5. Current knowledge on HYOU1, MANF, PDIA4, DNAJB11, and CALR

HYOU1 is known for maintaining homeostasis and limiting cell death (especially neurons) [301]. In mononuclear phagocytes, its expression not only enhances survival after acute lung injury but also promotes TLR-mediated activation of macrophages [302,303]. The role played by HYOU1 in fibrosis is less clear. On the one hand, it contributes to UPR response towards proteinuria and diabetic nephropathy constituting a protective response against fibrosis [304]. On the other hand, it is enhanced in fibrotic kidneys and chronic kidney disease [305,306]. A first study reported its overexpression in injured synovium [307], while more recent data have associated its role in synovial fibroblasts with FMT and pro-inflammatory cytokine production [293]. These observations are consistent with our *in vitro* findings, in which HYOU1 was

upregulated in FLS under inflammatory and TGF- β stimulations, supporting its potential involvement in synovial inflammatory and fibrotic processes. Nevertheless, its precise involvement in synovitis remains to be determined.

MANF is a cytoprotective protein promoting neuronal survival [308]. It also acts as an immune modulator that plays a critical role in tissue repair in the retina by switching macrophages from pro- to anti-inflammatory phenotype [309,310]. MANF is therefore further described as an anti-inflammatory agent, being able to extend lifespan in flies and to prevent aged-related liver disease in old mice [311]. It inhibits inflammation by downregulating NF- κ B [290,312,313]. MANF is also considered as a suppressor for hepatic fibrosis [311,314]. In rheumatic diseases, it is upregulated in the peripheral white blood cells isolated from RA and from systemic lupus erythematosus patients, and it is highly expressed in FLS from proliferative synovium of rats and rabbits [290]. Our study highlighted its overexpression and localization in the sublining of human synovitis, together with its induction under pro-inflammatory and TGF- β stimulations. Although these findings suggest a potential contribution to synovial pathology, its precise role in human synovium, particularly regarding proliferation, fibrogenesis, and inflammatory signaling, remains to be clarified.

We also observed that PDIA4 was overexpressed *in vitro* by human FLS, particularly under pro-inflammatory conditions, and even secreted in prolonged ER stress. Its potential role in pro-inflammatory pathologies has also been demonstrated, as this protein exhibits a strong correlation with various infiltrating immune cells, including monocytes, tumor-associated macrophages, and neutrophils within the glioma microenvironment [315]. Lung fibroblasts obtained from patients affected with chronic obstructive pulmonary disease exhibits higher levels of PDIA4 [316]. Protein disulfide isomerases (PDI) have been implicated in intestinal inflammation, as PDIA6 distribution in the colonic epithelium is increased in Crohn's disease (CD) compared to non-CD cases [317]. Similarly, PDIA4 is overexpressed in inflamed colonic mucosa from pediatric inflammatory bowel disease patients compared to uninfamed mucosa and controls [318]. Studies suggest that PDIA4 is involved in adipose tissue [319] and skeletal muscle [320] inflammation. A recent study suggests that it acts as a negative regulator of arthritis onset [321].

DNAJB11 is a cofactor of BiP and HSP90B1 proteins and it enhances their ATPase activity [322]. Presence of monoallelic mutations in DNAJB11 gene are responsible for an atypical form of autosomal-dominant polycystic kidney disease (ADPKD-DNAJB11) associating non-

enlarged polycystic kidneys and chronic interstitial fibrosis [323,324]. In our study, DNAJB11 was overexpressed *in vivo* in CIS mice synovial membranes and *in vitro* by human FLS under TGF- β stimulation. To our knowledge, this is the first study to describe its overexpression in the synovium.

CALR is a well-known chaperone whose soluble form may play a pathogenic role in autoimmune diseases [273]. High levels of CALR were previously detected in synovial cells from RA patients [325] and constitute a potential stromal-derived antigen driving RA [326]. CALR is also described as a promotor of apoptosis resistance of RA FLS through Bcl-XL and Mcl-1 expression [296]. Expression profile of OA and RA synovial tissues established by proteomics highlighted CALR in the whole proteome [327]. In our study, we confirmed the overexpression of CALR in RA synovitis but also in FLS under ER stress leading to its secretion once ER stress is prolonged. Beyond its established role in promoting angiogenesis in RA through NO signaling pathway [325], these findings suggest that CALR may represent a potential contributor to the development and maintenance of synovitis.

7.6. HYOU1 and PDIA4 are regulated through the IRE1 α branch of the UPR in FLS

Several studies support branch-specific regulation of ER chaperones by the UPR. In our model, pharmacological inhibition experiments indicated that HYOU1 and PDIA4 are regulated downstream of the IRE1 α and ATF6 branches in OA FLS.

Regarding the IRE1 α /XBP1 axis, transcriptomic analysis from Jiao *et al.* in human neuronal cell lines identified ER stress-related gene networks enriched in UPR signaling [328]. Within these networks, HYOU1, PDIA6 (not PDIA4), and DNAJB11 clustered with the IRE1 α /XBP1 pathway, supporting transcriptional regulation of these chaperones by this branch of the UPR [328]. In our results, inhibition of the IRE1 α pathway in FLS also modulated HYOU1 expression. Consistently, in hepatitis B models, activation of IRE1 α /XBP1s as well as tunicamycin-induced ER stress were shown to enhance MANF transcription [329].

Finally, involvement of the ATF6 pathway in ER chaperone regulation has also been described. Activation of ATF6 was associated with increased expression of PDI and CALR in Chinese Hamster Ovary cells [330], consistent with earlier observations by Okada *et al.* [331]. Conversely, co-culture of mucosal cells with *H. pylori* resulted in reduced ATF6 expression and a concomitant suppression of HYOU1 transcription [332]. In agreement with these

findings, our data indicate that HYOU1 and PDIA4 expression is also modulated downstream of the ATF6 pathway in FLS.

Regarding the PERK pathway, Okada *et al.* [331] reported that tunicamycin-induced transcription of PDIA4 was markedly reduced in mammalian cells carrying a homozygous mutation at the eIF2 α phosphorylation site, rendering them unable to activate PERK signaling. In line with this, the activation of ATF4 (a downstream mediator of PERK) was associated with increased expression of the chaperone proteins HYOU1 and CALR in an osteoblastic cell line [333]. However, in our model, pharmacological inhibition of PERK, confirmed by reduced EIF2 α phosphorylation, did not lead to detectable modulation of ER stress proteins in OA FLS.

To the best of our knowledge, this study is the first to investigate the regulation of ER stress proteins by selective inhibition of the major UPR branches in human synovial fibroblasts.

7.7. PDIA4 regulates proliferative responses in OA synovial fibroblasts via TGF- β signaling

In this study, we demonstrated that HYOU1, MANF, PDIA4, DNAJB11, and CALR were modulated within TGF- β and/or TNF- α stimulated OA FLS. OA FLS can transdifferentiate into myofibroblasts, characterized by α SMA expression and contributing to fibrotic remodeling [334]. The link between ER stress and EMT is well established, especially in peritoneal mesothelial, renal and alveolar epithelial cells [335–339]. These observations suggest that ER stress may more broadly contribute to fibrogenic cellular transitions, including FMT. This mesenchymal activation under ER stress involves the canonical SMAD2/3 pathway, as described in murine models of fibrosis [336], together with MAPK [338] and PI3K/AKT[340] signaling pathways activated in alveolar epithelial cells undergoing EMT (Figure 52). Even though the most powerful driver of this transition in response to ER stress is TGF- β [341], non-TGF- β pathways such as Wnt/ β -catenin [336] and Src kinase [338] have also been implicated.

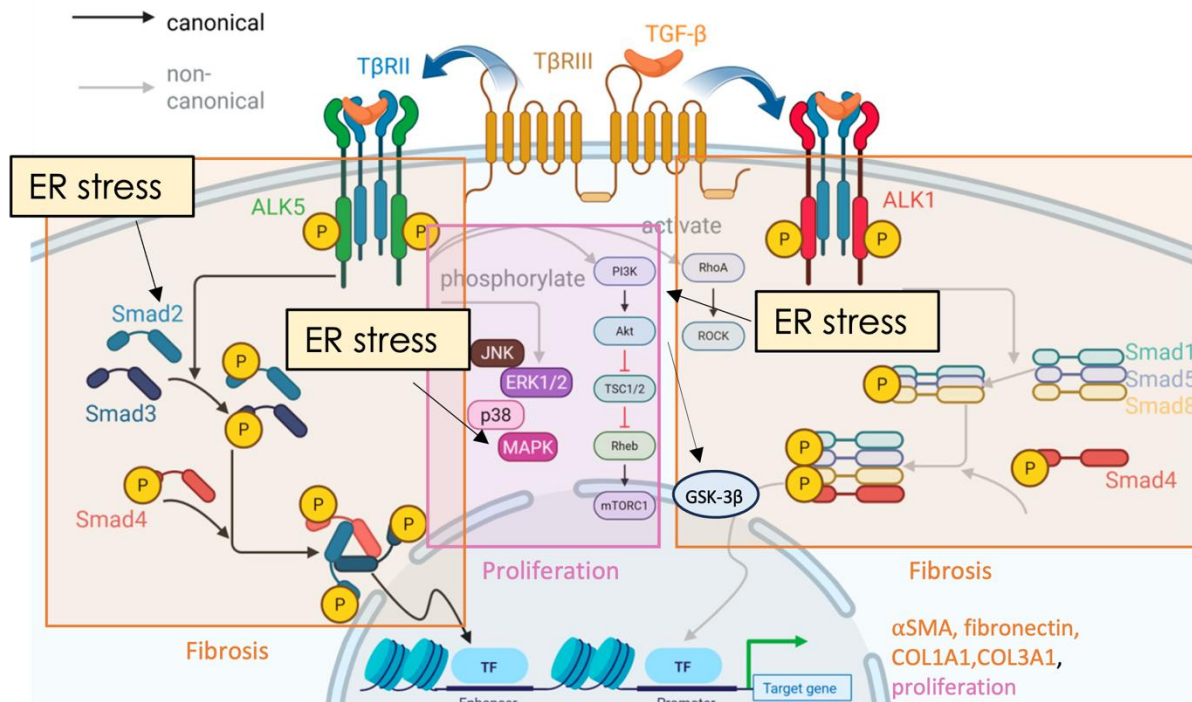


Figure 52. ER stress modulation of TGF-β-mediated FMT and proliferation pathways. ER stress promotes FMT and proliferation by activating multiple branches of TGF-β signaling. Canonical activation occurs through the Smad2/3 pathway, while non-canonical cascades include MAPK and PI3K/AKT, with downstream effectors such as mTORC1 or GSK-3β. These converging signals drive the transcription of pro-fibrotic genes, including α-SMA, fibronectin, COL1A1, and COL3A1 and fibroblasts proliferation. ER: endoplasmic reticulum; TGF-β: transforming growth factor-β; FMT: fibroblast–mesenchymal transition; MAPK: Mitogen-activated protein kinase. Adapted from [191].

A relationship between ER stress and FMT within the synovium has begun to emerge, as described in rat models of immobilization-induced joint contracture, suggesting that ER stress may play a role in driving fibroblast plasticity [342]. In line with this, an association of HYOU1, MANF and FMT markers in RA FLS has also been reported, although similar associations had not yet been described for the other ER stress proteins [290,293]. In this work, we were unable to demonstrate an association between ER chaperone depletion in FLS previously stimulated with TGF-β and modulation of α-SMA expression. Accordingly, under the experimental conditions tested, these ER stress proteins do not appear to be directly involved in FMT in OA FLS.

Following an initial investigation into fibrosis, it was found that ER stress proteins of our study had no direct effect on this process. We therefore focused on their potential role in driving an inflammatory state of FLS, reminiscent of an inflammatory pannus, by assessing their capacity

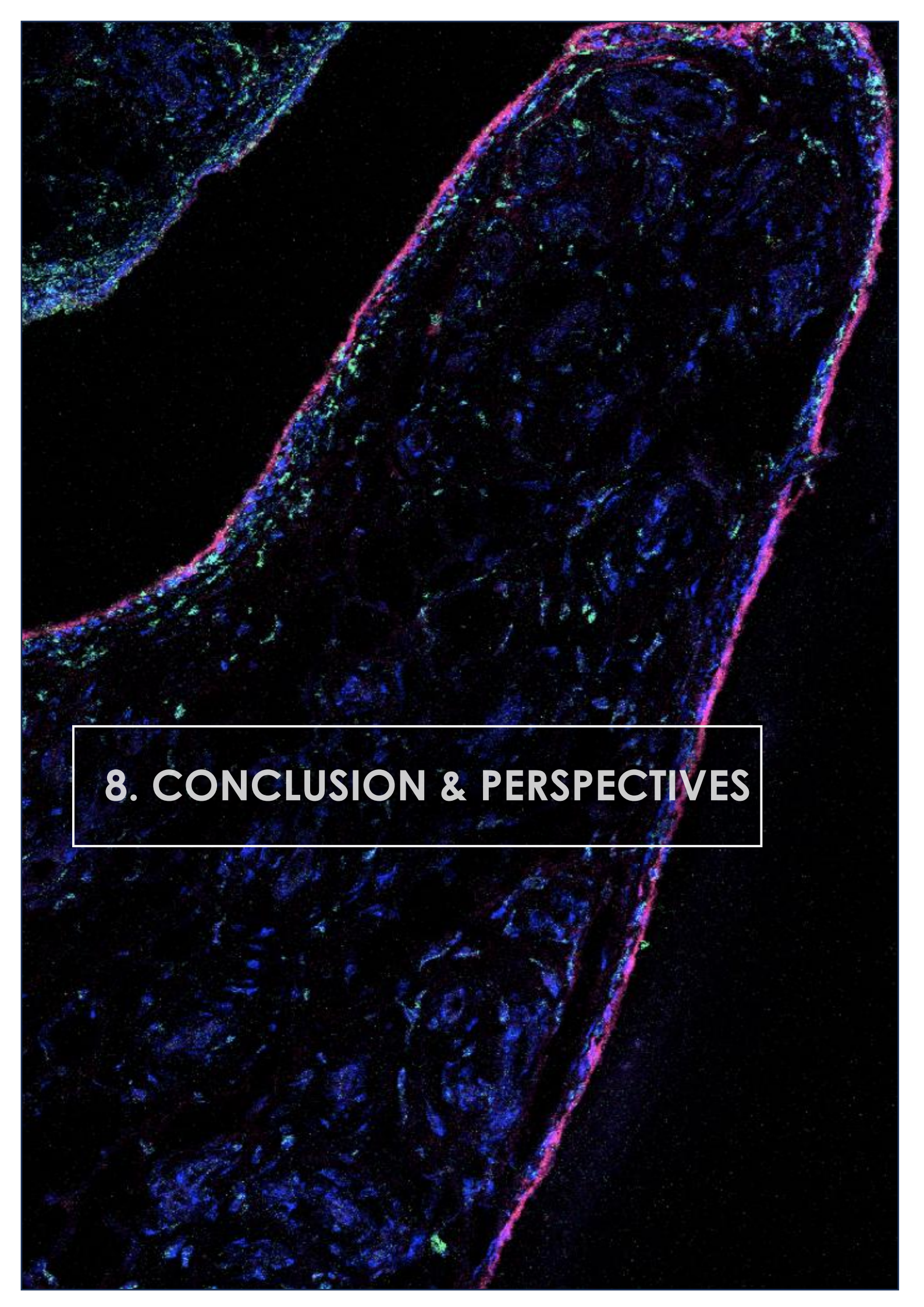
to induce IL-6 secretion. There is increasing evidence that ER stress promotes the secretion of chemokines and cytokines, thereby facilitating immune cell recruitment into tissues and enhancing their inflammatory activity [343,344]. These effects are largely mediated through NF- κ B signaling, which functions as a central pathway orchestrating pro-inflammatory responses [343]. In addition, ER stress has been identified as a trigger of inflammasome activation, as described in pancreatic β cells [345]. At the fibroblast level, ER stress has been shown to induce IL-6, CCL5, and thymic stromal lymphopoietin (TSLP) secretion by asthmatic lung fibroblasts, which in turn activate multiple immune cell populations, including dendritic cells, T lymphocytes, mast cells, and innate lymphoid cells [346]. Similarly, tunicamycin-induced ER stress markedly increased the expression of pro-inflammatory mediators such as IL-1 β and TNF- α in cultured RA FLS [280]. In line with these findings, ER stress has been directly linked to enhanced cytokine production in RA FLS, as reflected by increased secretion of IL-6, IL-8, and TNF- α [269,297]. Furthermore, increased levels of citrullinated BiP at the surface of RA-FLS and macrophages act as a receptor for ACPA, thereby enhancing NF- κ B-dependent inflammatory activation. Beyond its cell surface-associated role, extracellular BiP also displays pro-inflammatory activity, as its exogenous form enhances IL-17, TNF- α , and IL-23 production while reducing IL-10 in RA synovial mononuclear cells [277]. While our *in vitro* observations showed increased expression of ER stress proteins under inflammatory conditions, preliminary data suggested that these proteins might functionally contribute to inflammatory signaling. In particular, MANF has been proposed to exert anti-inflammatory effects, whereas HYOU1 has been implicated in cytokine production by RA FLS [293], PDIA4 has been linked to chronic inflammatory diseases [316,318] and CALR has been reported to support RA FLS survival [296]. Nevertheless, depletion of HYOU1, MANF, PDIA4, or CALR did not alter IL-6 secretion. Similarly, DNAJB11 knockdown had no effect, which is consistent with its lack of upregulation under pro-inflammatory conditions. These results should be interpreted in the context of our experimental design. Our analyses were restricted to FLS and did not include other synovial cell populations such as MLS. In addition, inflammation was assessed using a single readout, IL-6, chosen as a robust marker of intrinsic FLS activation, whereas other mediators such as IL-1 β , IL-8, or MMPs primarily reflect immune cell recruitment rather than fibroblast-driven inflammation (cf section 3.3.2). Moreover, inflammatory stimulation was limited to TNF- α , although other cytokines relevant to synovitis could also be considered. Importantly, our *in vitro* hypothesis was based on the observation that ER stress proteins are upregulated under TNF- α stimulation, and not on the assumption of a direct causal role in inflammatory processes.

This prompted us to explore whether ER stress proteins contribute to another key hallmark of synovitis, i.e.: FLS proliferation. Notably, HYOU1 [347], MANF [348], PDIA4 [349], DNAJB11 [350], and CALR [351] have already been reported to exert pro-proliferative effects in tumor cells, supporting survival and invasiveness. In addition, CALR has been specifically described as anti-apoptotic agent for RA FLS [296], whereas MANF has been reported to exert anti-proliferative effects on these cells [290]. Although preliminary and published in non-indexed journal, Zhao *et al*, recently reported that inhibition of the PDI family, including PDIA4, attenuated FLS proliferation of RA [352]. Induction of ER stress in lung fibroblasts has been associated with activation of the PI3K/AKT signaling pathway, which promotes fibroblast proliferation [353]; accordingly, silencing of XBP1 or its upstream sensor IRE1 α has been shown to reduce endothelial cell proliferation through suppression of PI3K/AKT/GSK3 β phosphorylation [354]. However, whether they play a similar role in OA fibroblast proliferation remained unknown. Here, we have demonstrated that depletion of PDIA4 was associated with a reduction in FLS proliferation. These findings suggest that PDIA4 may be involved in mechanisms supporting synoviocyte expansion, rather than directly regulating fibrotic or inflammatory processes in OA synovitis. However, this observation remains limited to the conditions tested and does not preclude additional roles for PDIA4 in other cellular contexts or inflammatory environments.

Taken together, while our data provide insights into the potential contribution of ER stress-related proteins to synoviocyte biology, several limitations must be acknowledged when interpreting these findings.

First, we must point out the lack of human healthy controls across all experiments, which limits the interpretation of baseline expression. To overcome this lack, we used the CIS mouse model. However, this model only partially mimics human synovitis and does not reproduce its cellular heterogeneity. Besides, all our analyses were based on an inflammatory histological scoring system (modified Tak score), which is not fully quantitative and may overestimate inflammation in cases where a single lymphoid aggregate is present despite otherwise low synovitis. Therefore, caution is warranted in interpreting the correlations drawn from this scoring system. Regarding IMC, the limited number of OA samples prevented the establishment of a true inflammatory continuum, and the insufficient CD90 signal did not allow us to distinguish CD90⁺CD34⁺ from CD90⁺CD34⁻ populations. In addition, the IMC cohort (cohort 2) should be considered as an intermediate and exploratory dataset bridging cohorts 1 and 3, as it includes

only two OA biopsies and one RA sample, thereby limiting the robustness and generalizability of these observations. In IF, the analysis was mainly restricted to PDIA4 because most antibodies were raised in rabbit, precluding multiplex staining of several ER stress proteins on the same section (although a rabbit-on-rabbit technique allowed limited combinations). *In vitro*, the range of stimuli was restricted, as ER stress was only induced by tunicamycin, which does not recapitulate the full complexity of the *in vivo* environment where ER stress coexists with oxidative stress, hypoxia, and mechanical cues. Furthermore, ER stress protein secretion was confirmed only under prolonged ER stress; proteins were not detected in supernatants after TNF α or TGF- β stimulation, highlighting the need for more sensitive approaches such as ELISA. In addition, several of our findings are based on spatial and expression correlations (e.g., PDIA4 with CD55⁺ vs CD34⁺ FLS) rather than direct mechanistic proof, which prevents definitive causal inference. Similarly, our conclusions regarding phenotypic transitions rely in part on previously published single-cell studies. However, we did not perform single-cell sequencing ourselves, which would have provided higher resolution on FLS subsets and their dynamic transitions. Finally, the small sample size did not allow for robust statistical analyses.

A fluorescence microscopy image showing a biological specimen, possibly a cell or tissue section. The specimen is stained with two different dyes: a blue dye (likely DAPI) that highlights the nuclei and a red dye (likely a membrane or cytoplasmic stain). The blue staining is concentrated in the nuclei, while the red staining outlines the cell boundaries and some internal structures. The background is dark, making the stained areas stand out.

8. CONCLUSION & PERSPECTIVES

ER stress proteins are dynamically redistributed during synovial inflammation, shifting from CD55⁺ lining cells to CD34⁺ sublining fibroblasts and appearing predominantly associated with fibroblast populations rather than immune cells. Among them, PDIA4 seems to be involved in synoviocyte proliferation and in the expansion of CD34⁺ fibroblasts within the sublining, suggesting a potential role in synovial remodeling during OA synovitis.

Consistent with this overall pattern, our data show that ER stress proteins in OA are initially restricted to the lining layer and are then overexpressed in the sublining as inflammation worsens, with a localization throughout the whole synovium (that has lost its architecture) in RA. These findings were validated in the CIS mouse model, which highlighted the association of several ER stress proteins with inflammatory and pro-fibrotic pathways. By combining IMC and IF, we confirmed that these proteins are expressed by CD55⁺ FLS and CD68⁺ MLS in the lining layer of NIN OA synovium. In IN OA, their expression shifted toward CD34⁺CD31⁻ fibroblasts in the sublining, a population with profibrotic features, consistent with phenotypic changes associated with deeper synovial localization. These findings suggest that FLS may represent a major cellular source of ER stress responses within the OA synovium under the conditions tested, compared to MLS or lymphocytes.

In vitro, we showed that most of these proteins are expressed at baseline to support homeostasis, but are markedly upregulated under ER stress, inflammatory, and fibrotic conditions, with some of them being secreted and thereby acquiring potential pro-inflammatory properties. Among them, HYOU1, MANF, PDIA4, DNAJB11, and CALR emerged as particularly relevant, in line with their known involvement in other inflammatory and fibrotic diseases. In our experimental conditions, functional assays in FLS indicate that PDIA4 may participate in proliferative processes, and could therefore contribute to synovitis and OA progression.

This study provides a spatial and functional characterization of ER stress proteins in human inflamed synovium, suggesting a link between ER stress and fibroblast plasticity, and identifying a potential association between ER chaperones and proliferative processes in OA synovitis.

Future studies should clarify the specific contribution of each FLS subset. In particular, isolating CD55⁺ and CD34⁺CD31⁻ FLS by FACS would make it possible to: i) explore the relationship between CD34⁺CD31⁻ FLS enrichment and synovial inflammation according to the Tak score; ii) functionally compare CD55⁺ and CD34⁺ FLS in response to key ER stress-

related factors, iii) assess whether ER stress proteins modify their phenotype from CD55⁺ into CD34⁺CD31⁻ cells.

We should also further investigate CD68⁺ MLS in OA synovium, as they appear to express the ER stress proteins of interest. Isolating them for *in vitro* studies would help clarify their potential interactions with FLS.

In addition, future work should determine whether ER stress proteins directly dysregulate UPR signaling—leading to its chronic overactivation and bias—or whether, conversely, activation of specific UPR branches drives their overexpression and eventual secretion into the extracellular space. Indeed, the secretory dimension also warrants attention: ER stress proteins released should be quantified in synovial fluid and blood (e.g., by ELISA) in larger OA and RA cohorts, to assess whether, as reported for BiP, CALR, and HSP90B1 in RA [276–278], they act as autoantigens and participate in triggering inflammatory responses in OA. Such analyses would also determine their potential as novel biomarkers for disease activity, prognosis, or treatment response.

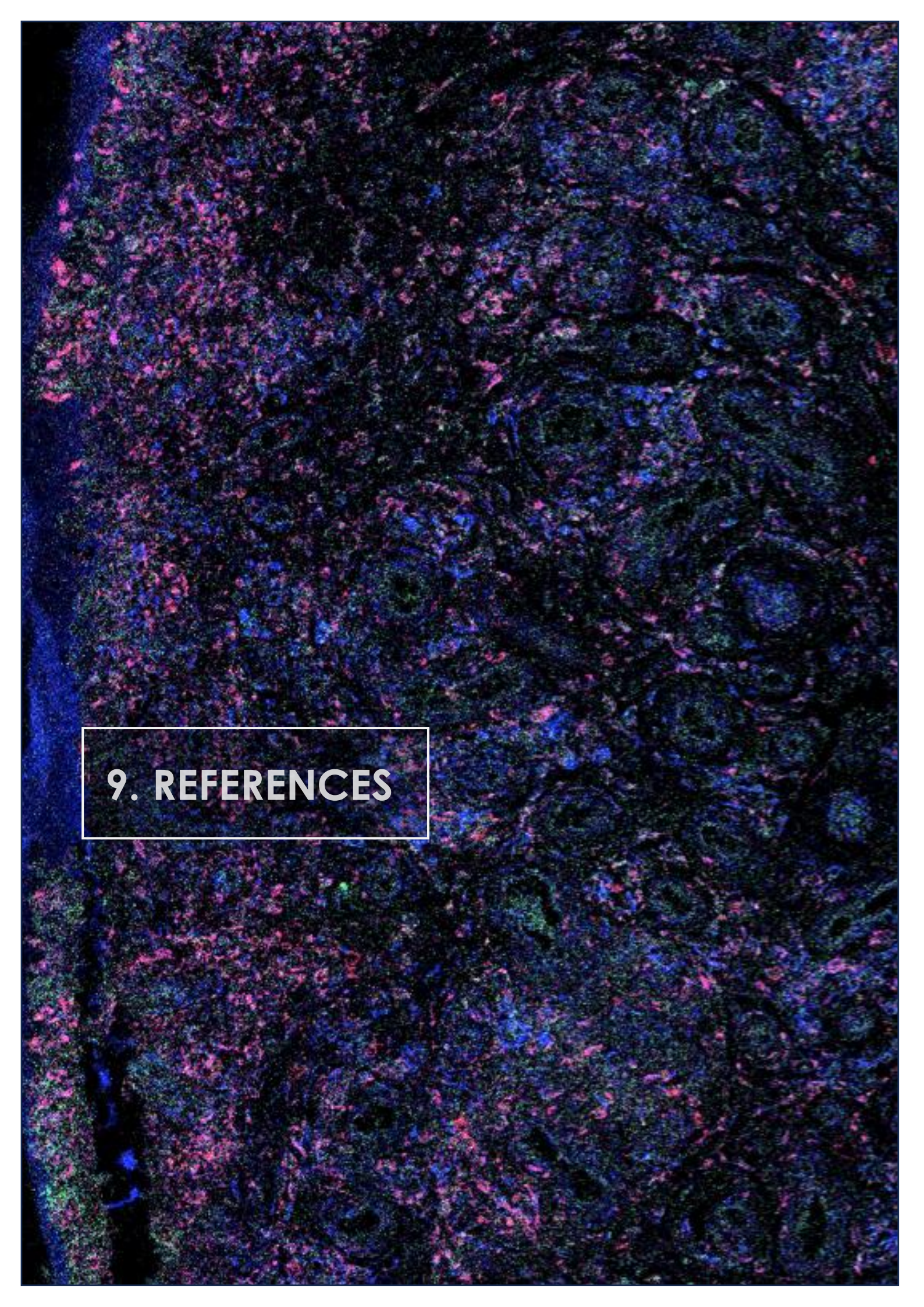
Finally, functional studies could leverage pharmacological inhibitors targeting HYOU1, MANF, PDIA4, DNAJB11, and CALR to evaluate their impact on synovial inflammation and fibrosis. Among them, HYOU1, PDI and CALR small-molecule inhibitors have already been implemented [293,352,355,356]. Conditional knockout mouse models (e.g., specific deletion of PDIA4) would provide *in vivo* confirmation of their role in synovitis. Overall, given its involvement in OA fibroblast proliferation, PDIA4 in particular appears to be a promising future therapeutic target.

Beyond these mechanistic insights, our findings also raise broader clinical perspectives. The identification of ER stress proteins as potential contributors to synovial activation suggests that ER stress could represent a previously underappreciated therapeutic axis in OA and inflammatory joint diseases. Targeting ER stress pathways may therefore complement current strategies that mainly focus on inflammatory cytokines or pain control.

Interestingly, several metabolic agents already evaluated in OA may partly exert their effects through modulation of ER stress pathways. Experimental studies indicate that metformin can attenuate ER stress–induced apoptosis, modulate UPR pathways, and restore protein translation through mTOR/4E-BP1 signaling [357–359]. Similarly, GLP-1 receptor agonists have been shown to reduce ER stress markers, inhibit PERK/ATF4/CHOP signaling, and limit apoptosis in various cell types, including chondrocytes [360–362]. However, given that synovial

pathology is primarily characterized by altered fibroblast proliferation rather than excessive apoptosis, the relevance of these anti-apoptotic effects in this context remains uncertain. These findings nonetheless support the idea that modulation of ER stress pathways may contribute to the biological effects of these agents on OA.

Taken together, these findings support the concept that part of the beneficial effects of metabolic therapies in OA may be mediated through the modulation of ER stress and associated apoptotic pathways. In this context, the potential role of PDIA4 in FLS proliferation raises the possibility that more specific pharmacological targeting of ER stress regulators could limit pathological synovial hyperplasia. If validated in vivo, such approaches could represent a novel disease-modifying strategy aimed at controlling synovial activation and disease progression.



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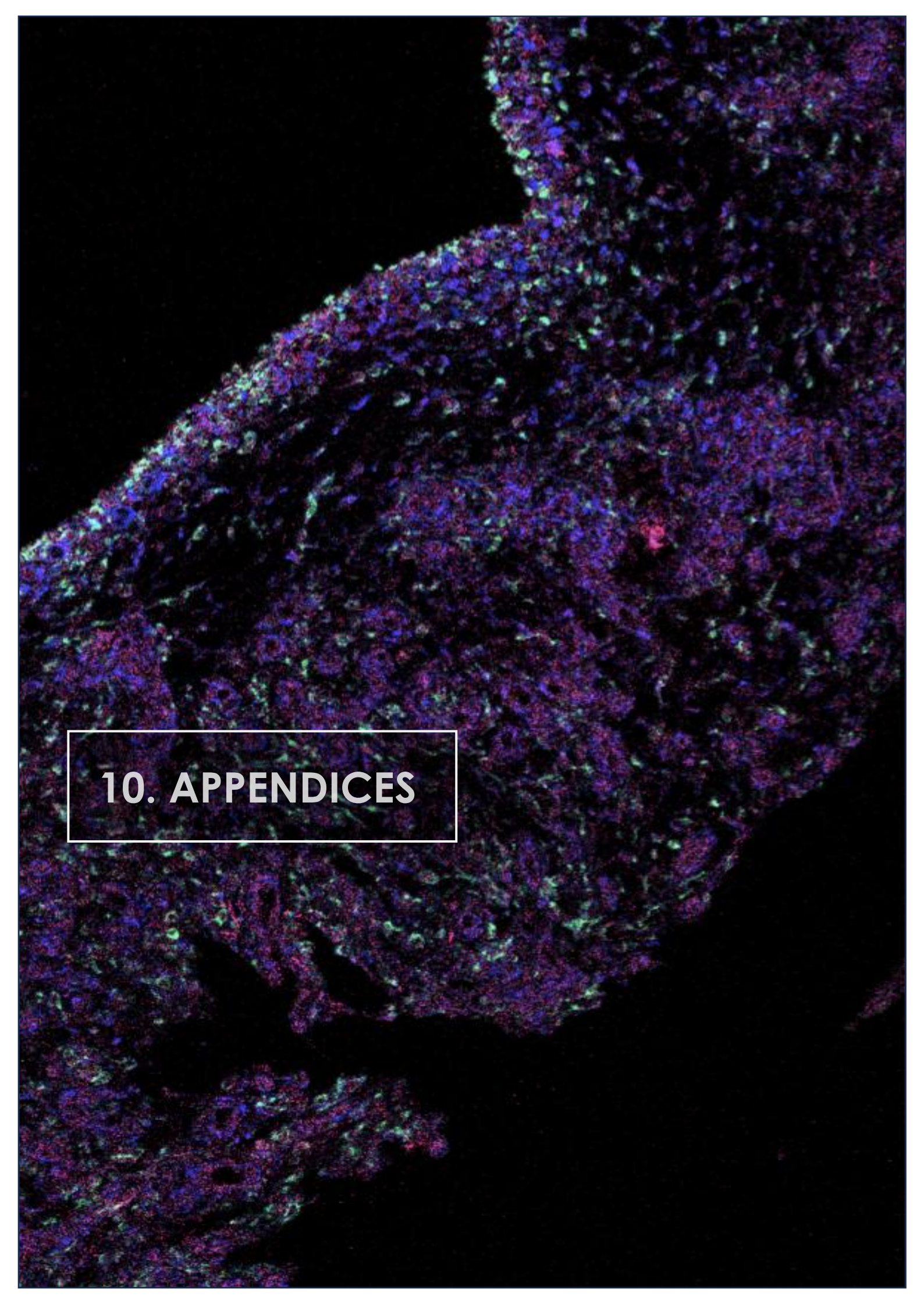
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10. APPENDICES

The first of these is the fact that the system is not a simple one. It is a complex system, and as such, it is not possible to understand it by looking at its parts in isolation. The system is a whole, and its behavior is determined by the interactions between its parts. This is a fundamental principle of systems thinking, and it is one that is often overlooked in traditional engineering approaches.

The second of these is the fact that the system is not a static one. It is a dynamic system, and its behavior changes over time. This is another fundamental principle of systems thinking, and it is one that is often overlooked in traditional engineering approaches.

The third of these is the fact that the system is not a linear one. It is a non-linear system, and its behavior is not predictable by simple linear models. This is another fundamental principle of systems thinking, and it is one that is often overlooked in traditional engineering approaches.

The fourth of these is the fact that the system is not a closed one. It is an open system, and it interacts with its environment. This is another fundamental principle of systems thinking, and it is one that is often overlooked in traditional engineering approaches.

The fifth of these is the fact that the system is not a deterministic one. It is a probabilistic system, and its behavior is not predictable with certainty. This is another fundamental principle of systems thinking, and it is one that is often overlooked in traditional engineering approaches.

The sixth of these is the fact that the system is not a single one. It is a multi-actor system, and its behavior is determined by the interactions between its many actors. This is another fundamental principle of systems thinking, and it is one that is often overlooked in traditional engineering approaches.

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**Submitted: Spatial and Functional Characterization of ER Stress Proteins Links
Fibroblast Plasticity and Proliferative Processes in Human Osteoarthritic Synovitis**

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ABSTRACT

A strong crosstalk exists between endoplasmic reticulum (ER) stress and synovitis. Beyond their canonical role in protein folding, ER stress chaperones may promote inflammation, cell survival, and fibroblast activation under pathological conditions. This study aimed at localizing and quantifying 11 ER stress proteins (BiP, HYOU1, MANF, PDIA4, GANAB, HSP90B1, TXNDC5, DNAJB11, LMAN1, ERP29, CALR) in human inflamed synovial membranes and at investigating their expression in fibroblast-like synoviocytes (FLS) under ER stress, pro-inflammatory, or pro-fibrotic stimuli.

By immunohistochemistry, on a first cohort of formalin-fixed paraffin-embedded (FFPE) biopsies obtained from patients with osteoarthritis (OA), chronic pyrophosphate arthropathy (CPPA), and rheumatoid arthritis (RA), these ER chaperones were primarily localized to the lining in low-grade inflammation ($Tak < 4$) and expanded to the sublining under high inflammatory conditions ($Tak \geq 4$), with a widespread distribution in RA.

Imaging mass cytometry, applied to a second cohort of FFPE tissue samples collected from patients diagnosed with OA and RA, revealed the co-expression of ER stress proteins with CD55⁺ FLS in the lining and their progressive infiltration into the sublining along with CD34⁺CD31⁻ FLS during inflammation.

These observations were confirmed by immunofluorescence on a larger cohort of OA patients. As inflammation progresses, there is a loss of co-expression with CD55 in the lining, accompanied by a gradual shift towards co-expression with CD34 in the sublining.

In vitro, ER stress proteins, particularly BiP, HYOU1, MANF, PDIA4, HSP90B1, LMAN1, CALR, and DNAJB11 are overexpressed in human OA FLS following ER stress, pro-inflammatory or pro-fibrotic stimulation, with BiP, PDIA4, HSP90B1, ERP29 and CALR also being secreted. PDIA4 emerged as a central player: its depletion significantly impaired FLS proliferation and migration, highlighting a direct role in driving synovitis.

This study provides the first spatial and functional characterization of ER chaperones in human arthritic synovium, linking ER stress to fibroblast plasticity, inflammation, and fibrosis.

INTRODUCTION

The synovial membrane is a connective tissue that surrounds joints and contributes to their homeostasis and lubrication. It is composed of two layers: the lining and sublining. The lining is a thin layer in contact with synovial fluid and contains two synoviocyte types: (i) macrophage-like synoviocytes (MLS, 75%) with phagocytic activity and macrophage markers and (ii) fibroblast-like synoviocytes (FLS) secreting collagen and hyaluronan. The sublining is less compact and is mainly composed of blood vessels, fibroblasts and adipocytes, with few resident interstitial macrophages and lymphocytes in a collagenous extracellular matrix [161].

Synovitis is characterized by FLS proliferation and hyperplasia of the lining cells, with immune cell infiltration, neoangiogenesis and fibrosis. These characteristics appear in both osteoarthritis (OA) and rheumatoid arthritis (RA) but are more pronounced in the latter [161,363]. Inflammation rapidly alters lining morphology and MLS orientation, abrogating cell–cell contacts [162]. Simultaneously, FLS infiltrate the sublining and differentiate into pro-inflammatory and pro-fibrotic subsets [162,181]. Single-cell RNA sequencing studies have identified key markers distinguishing FLS subsets in synovial tissue. CD55 marks lining FLS, which are central to synovial fluid production and play a homeostatic role through their barrier function, whereas CD34 characterizes sublining FLS, which exhibit distinct responses to cytokine signaling and express genes linked to fibrosis and angiogenesis in pathological conditions [182,204,205,364].

The endoplasmic reticulum (ER) ensures proper protein folding and trafficking through the activation of chaperones. When misfolded proteins accumulate, the unfolded protein response (UPR), involving IRE1, PERK, and ATF6, is triggered to restore homeostasis [365,366]. Sustained ER stress can promote inflammation via ROS, calcium release, and NF- κ B activation [367]. Moreover, chaperones may acquire pro-inflammatory roles when exposed on the cell surface or secreted, enhancing cytokine production and promoting FLS proliferation and migration [273,287].

Evidence indicates that ER stress pathways are directly implicated in the pathogenesis of synovitis [11,263–265]. Building on this concept, we recently used mass spectrometry to identify ER stress proteins in synovial membranes from patients with OA, chronic pyrophosphate arthropathy (CPPA), and RA, and exhibiting a continuous degree of inflammation [13]. Expression level of eleven ER stress proteins: BiP (binding immunoglobulin

protein), HYOU1 (hypoxia up-regulated 1), MANF (mesencephalic astrocyte-derived neurotrophic factor), PDIA4 (protein disulfide isomerase A4), GANAB (α -subunit of glucosidase II), HSP90B1 (heat shock protein 90 beta family member 1), TXNDC5 (thioredoxin domain containing 5), DNAJB11 (dnaJ heat shock protein family member B11), LMAN1 (lectin mannose-binding 1), ERP29 (endoplasmic reticulum protein 29) and CALR (calreticulin) were strongly correlated to the histological inflammatory score (HIS) based on hyperplasia, macrophages, lymphocytes, plasmocytes and neutrophils infiltration in synovial membranes [13].

In this study, we aimed to characterize the spatial distribution of ER stress proteins within inflamed human synovial membranes and in a mouse model, with a particular focus on CD55⁺ and CD34⁺ FLS subpopulations using imaging mass cytometry (IMC) and immunofluorescence (IF) on two distinct cohorts. Moreover, we investigated the potential role of these ER stress proteins in fibroblast proliferation using primary FLS.

This study provides the first spatial characterization of ER stress proteins across distinct fibroblast subpopulations within the synovitis, revealing a dynamic and progressive pattern of expression within CD55⁺ and CD34⁺ FLS, which correlates with the inflammatory grades.

METHODS

Synovial biopsies

All experiments were undertaken with patient material complying with the regulations and ethical guidelines of the University Hospital of Liège (CHU) and the Catholic University of Louvain (UCLouvain), Belgium, and were approved by their respective ethical committee (for CHU cohorts- ref: 2017/147, for UCLouvain cohort- ref:2013/24MAI/293) and the biobank research committee (CHU - BB190058, UCLouvain –BB190044). Synovial biopsies were obtained via needle arthroscopy or small joint ultrasonography guided biopsy and were embedded in paraffin for immunohistochemistry (IHC) analysis. Three cohorts (Supplementary Table 1a) were included: i) **cohort 1** (n=24) for IHC from the Centre Hospitalo-Universitaire de Liège (CHU Liège) naïve of any treatment [13]; for this cohort, no statistically significant differences were observed between groups for gender, age or BMI. ii) cohort 2 from UCLouvain (n=3) used for imaging mass cytometry (IMC); iii) cohort 3 (n=25) from CHU of Liège for immunofluorescence (IF). For each cohort, chosen biopsies collectively illustrated a continuous inflammatory gradient according to a modified version of the Tak score, modified by excluding

vascular parameters (hereafter referred to as “Tak score” - Supplementary Table 1b), which is based on hyperplasia (hy; 0–4 score), lymphocytes (ly; 0–4 score), plasmocytes (pl; 0–4 score), and neutrophils (PMN; 0–3 score) infiltration [211]. Hematoxylin eosin stained sections were scored by anatomopathologists (EB & PD) for each histological feature providing the Tak score. Based on this scoring system, “non-inflammatory (NIN) OA” was defined as OA samples with low Tak scores (Tak < 4), while “inflammatory (IN) OA” corresponded to samples with higher Tak scores (Tak ≥ 4).

Collagenase-induced synovitis mouse model

All animal experimentation procedures were approved by the local ethical committee (LA1610002; #14-1721 University of Liège, Liège, Belgium). The collagenase-induced synovitis (CIS) mouse model was performed as previously described by Deroyer *et al* [200]. Briefly, 8-week-old C57BL6 male mice were anesthetized using isoflurane. Only male mice were used in this study, as OA is known to be more robustly induced in males than in females in experimental models, even though the human tissue cohort primarily consisted of aging women (see Supplementary table 1a) [368,369]. Intra-articular injection was performed randomly into knee joint cavities with collagenase VII for CIS generation (1U per articulation, Sigma-Aldrich, St Louis, Michigan, USA) or NaCl 0.9% (control mice) two times, 2 days apart. Mice were sacrificed 1 week later, and joints were collected. Synovial membrane inflammation in CIS mice was confirmed by the overexpression of mRNA *Il1b* and *Il6* measured by RT-qPCR and synovial thickness [200]. Overexpression of *Acta2* mRNA also confirmed the initiation of fibrosis [200]. Investigators were not blinded to group allocation.

Immunohistochemistry analysis

IHC was performed on human and mouse synovial membranes. For human samples, tissues obtained from the ULiège cohort (Cohort 1) were fixed in 4% paraformaldehyde for 24 hours (h), then treated in 70% (v/v) ethanol and embedded in paraffin. For mouse samples, the knee joints were fixed in 4% paraformaldehyde for 24h, followed by decalcification in EDTA for 15 days. They were then dipped in 70% (v/v) ethanol and embedded in paraffin. IHC was then performed on the slide after heating, dewaxing, and antigen retrieval using a steamer for 10 minutes in target retrieval solution (Agilent, Santa Clara, California, USA). To block endogenous peroxidases and non-specific sites, slides were incubated with hydrogen peroxide and DakoReal antibody diluent (Agilent), respectively. For human FFPE, sections were incubated with a primary antibody (Supplementary Table 2) against chaperone proteins of

interest. For mouse model (CIS), only BiP, MANF, PDIA4, HSP90B1, CALR, DNAJB11 and LMAN1 antibodies could be used due to species compatibility. Each ER stress protein is referred to its gene name in Supplementary Table 2. Sections were then incubated with EnVision+System-HRP-labeled polymer (Agilent), and peroxidase was detected using the Liquid DAB+ Substrate Chromogen System (Agilent). Proteins of interest were localized, semi-quantified and normalized for the total number of cells using *QuPath* software [279].

Imaging mass cytometry

Imaging Mass Cytometry™ (IMC™) (Hyperion Imaging System, Standard BioTools™, California) was performed on FFPE tissue provided from 3 patients of the UCLouvain cohort (Cohort 2) as described in the Supplementary Table 1. These tissues were deparaffinized and then treated with antigen retrieval buffers. The sections were incubated with a mixture of metal-conjugated antibodies (Supplementary Table 2) and a DNA intercalating agent for nuclear staining. Each antibody was labeled with a unique metal isotope, allowing for specific recognition of its corresponding antigen and enabling multiplexed spatial protein profiling by imaging mass cytometry (CyTOF). The Hyperion Imaging System (Standard BioTools™, South San Francisco, USA) performed laser desorption of ionized isotopes on selected regions of interest of the synovial tissue. These ions were then separated according to their m/z ratio and quantified by the CyTOF instrument, providing information about the intensity and spatial distribution of our proteins of interest.

Multiparametric images obtained by IMC were further studied by the imaging platform (GIGA-Cell Imaging, ULiège). Raw imaging mass cytometry data in “.mcd” format were converted to the standardized “.ome.tiff” format using a custom Python script developed in-house, ensuring the complete preservation of spatial metadata and compatibility with downstream analysis tools. Tissue classification and nuclear segmentation were performed using QuPath (v0.5.1) on the standardized images. Tissue regions were segmented using a pixel classifier trained in QuPath to distinguish tissue from background based on DAPI fluorescence intensity, using a Random Forest algorithm. Nuclei were segmented using StarDist, integrated into QuPath via its dedicated extension. Segmentation was performed on the DAPI fluorescence channel, using a custom-trained StarDist model optimized for histological nuclear features. Using the resulting single-cell masks, Mass Spectrometry signal for 19 markers (Supplementary Table 2) were quantified on a per-cell basis within QuPath. The default threshold for each marker was defined based on the three OA samples, using the mean plus two standard deviations, after filtering out all cells with values exceeding 1.5 times the interquartile range. Downstream analysis was

performed using CytoMAP (v1.4.21). Clustering was conducted using a Self-Organizing Map (SOM) algorithm with *Nearest Neighbor (NN)* spatial context, and the optimal number of clusters was determined using the Davies–Bouldin index.

Immunofluorescence on formalin-fixed paraffin-embedded tissues

Formalin-fixed paraffin-embedded tissue slides from the Cohort 3 (CHU of Liège – 25 OA) were incubated overnight at 65°C. Slides were deparaffinized, followed by antigen retrieval using heat-induced epitope retrieval (HIER) with Target Retrieval Solution (Agilent). Endogenous peroxidase activity was blocked with H₂O₂ for 20 minutes. To reduce background and begin permeabilization, slides were incubated in a solution of 0.4% Triton X-100 (CAS-9036195; Sigma Aldrich) and 125 mM glycine. Slides were placed in a humidified chamber, and non-specific binding was blocked with Protein Block Serum-Free (Agilent, X0909). Three successive cycles of primary (Supplementary Table 2) and corresponding secondary antibody incubations were performed, each separated by permeabilization and blocking steps. Step 1: mouse anti-CD34 and rabbit anti-PDIA4 (or anti-CALR), followed by anti-mouse Alexa Fluor 594 (ab150116, Abcam) and anti-rabbit Alexa Fluor 532 (A11009, Thermo Fisher). Step 2: rabbit anti-CD55 followed by anti-rabbit Alexa Fluor 488 (ab150081, Abcam). Step 3: directly conjugated antibodies anti-CD68-Alexa Fluor 647 and anti-CD31-Alexa Fluor 750. On day 4, nuclear staining was performed with DAPI (1:1000) for 30 minutes. Slides were mounted with ProLong Diamond Antifade Mountant and stored at 4°C. Image acquisition was performed using a Leica Stellaris 8 confocal imaging system and proteins of interest were next localized and semi-quantified using *QuPath* software [279].

Cell culture and treatment

Synovial tissues were obtained prospectively by orthopaedic surgeons of the CHU Liège from patients who underwent prosthetic surgery for knee OA. The research ethics committee of our hospital, CHU of Liège, Belgium, approved the study (ref: 2023/4). To isolate human FLS from synovial membrane, the tissue was digested with collagenase overnight at 37°C. Cells were filtrated and resuspended in DMEM containing 10% foetal bovine serum (FBS). Cells were used between passages 3 and 7. Synovial membranes used for FLS isolation were provided from 60 patients: 45% females and 55% males with a median age of 67 (54-84) years and a median BMI of 34.5 (19.05-38.27) kg/m². ER stress was induced by tunicamycin (Tm; 1 µg/mL; Sigma aldrich) for 1, 3 or 7 days before supernatant and protein extract collection. For protein secretion in supernatants, FBS 1% was used instead of FBS 10%. Inflammation was induced

by TNF- α (10ng/mL, Peprotech, London, UK) for 1, 3 or 7 days. Pro-fibrotic response was induced by TGF- β (10ng/mL; ThermoFischer) for 1, 3 or 7 days. Cell extracts and supernatants were then collected. These stimulation time points were defined based on preliminary data from our laboratory on OA FLS [89,201] and supported by the literature, showing time-dependent induction of ER stress markers [280], pro-inflammatory cytokines (e.g., IL-1 β , IL-6) [89,281], and pro-fibrotic markers (e.g., α SMA, fibronectin) [89,201]. The UPR pharmacological inhibitors (MedChemExpress) of IRE1 α (4 μ 8C, 100 μ M; HY-19707), PERK (PERK-IN-2, 0.01 μ M; HY-135220) or ATF6 (Ceapin-A7, 0.5 μ M; HY-108434) were applied 4 h before a 1-day or 3-day stimulation with Tm or TGF- β , respectively. Diméthylsulfoxyde (DMSO) was used on controls. Protein extracts were collected for downstream analysis.

Immunofluorescence on fibroblast-like synoviocytes (FLS)

FLS were seeded on glass coverslips, stimulated by Tm (1 μ g/mL) for 3 days, and fixed with 4% paraformaldehyde for 10 minutes. Cells were then permeabilized and blocked using PBS supplemented with 1% BSA, 5% FBS, and 0.1% Triton X-100 for 30 minutes. Cells were then incubated overnight at 4 °C with primary antibodies against CD55 or CD34 (Supplementary Table 2). Coverslips were incubated for 1h with an Alexa Fluor 647-conjugated anti-rabbit secondary antibody (A-21235; Invitrogen). Nuclei were counterstained with DAPI (1:1000), and coverslips were mounted using ProLong Gold Antifade Mountant.

siRNA transfection

For RNA interference, the decreased expression level of HYOU1 (s20632), MANF (s15436), PDIA4 (s225164), DNAJB11 (s28581) or CALR (s115) was obtained by siRNA transfection (Life Technologies) using Lipofectamine™ 2000 (Thermo Fisher Scientific, Cat. No. 11668030). siRNAs were resuspended to 100 μ M stock solutions and diluted to 10 μ M working solutions. For transfection, siRNAs were diluted either 1:10 (HYOU1, DNAJB11) or 1:20 (MANF, PDIA4, CALR) in Opti-MEM™, while Lipofectamine™ 2000 was diluted 1:50 in Opti-MEM™, using equal volumes. After 5 min incubation at room temperature (RT), both solutions were mixed and incubated for 15 min to allow complex formation. The transfection mix was then added dropwise to the cells, resulting in a final 1:10 dilution in the culture medium. A Negative Control siRNA (siCtrl, Cat. No. 4390843) was used at matching concentrations for each condition, with separate controls corresponding to the 1:10 and 1:20 siRNA dilutions. FLS were seeded in 96-well plates and transfected for 24h prior to a 3-day TGF- β stimulation.

Viability and proliferation assays

FLS were transduced with siRNAs targeting HYOU1, MANF, PDIA4, DNAJB11, CALR or siCtrl, and were subsequently stimulated with TGF- β for 3 days. Cell viability was evaluated using the MTS assay (Promega, Madison, WI, USA), while cell proliferation was assessed using the BrdU incorporation assay (Abcam), following the manufacturers' instructions. Results are expressed as the percentage of viable (MTS assay) or proliferating (BrdU assay) cells. Proliferation and MTS assays were performed separately for HYOU1 and DNAJB11 on the one hand, and for MANF, PDIA4, and CALR on the other, as each protein group was compared with its own matched control condition at the corresponding concentration. Accordingly, their respective depletions were carried out and quantified in parallel but analyzed independently.

Migration assay

OA FLS were seeded in both 96-well plates (for migration analysis) and 24-well plates (for validation of PDIA4 depletion) and cultured in DMEM supplemented with 10% FBS until reaching full confluence. Three days post-seeding, the culture medium was replaced with low-serum medium (0.5% FBS), and cells were transfected in the morning with either control siRNA (siCtrl) or siRNA targeting PDIA4 (siPDIA4). Six hours later, cells were stimulated or not with TGF- β . For migration analysis, 96-well plates were processed using the IncuCyte Live-Cell Analysis System. Wound areas were defined using the IncuCyte plate map editor, and cell migration was monitored in real time over a period of 60h. Images were acquired automatically, and relative wound density (RWD) was quantified over time. Experiments were terminated after the 60h acquisition period. In parallel, cells cultured in 24-well plates were harvested for protein extraction to confirm the efficiency of PDIA4 knockdown by Western blot analysis.

Western blotting

FLS were lysed in RIPA buffer. Proteins from cell lysates were then separated onto polyacrylamide gels. Proteins were then transferred overnight on polyvinylidene difluoride membranes (Millipore, Tullagreen, Ireland), which were further blocked with 10% milk for 1h at RT. The blots were first incubated with the primary antibody (Supplementary table 2) for 3h at RT, washed, and then incubated with the horseradish secondary antibodies (anti-mouse or anti-rabbit; Cell Signaling Technology) for 45 minutes at RT. Proteins of interest were detected by chemiluminescent detection solution (ECL Western blotting substrate; Thermo Scientific, Rockford, USA). Glyceraldehyde-3-phosphate dehydrogenase (anti-GAPDH #G9545; Sigma)

and Heat Shock Protein (anti-HSP90 #13171-1-AP; Proteintech) were used as protein-loading controls for intracellular extracts, whereas Ponceau S stain (CAS-6226795; Sigma aldrich) was used for supernatants. Intensities were normalized using the corresponding loading control and further quantified using ImageJ software (version 1.53).

RT-qPCR

Total RNA was extracted using the NucleoSpin RNA kit (Macherey–Nagel, Düren, Germany). RNA was reverse-transcribed using the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA). cDNA was amplified by real-time PCR using the KAPA SYBR FAST system (Sopachem, Belgium) on a LightCycler 480 instrument (Roche Diagnostics, Germany). PCR efficiency $E = 10^{(-1/\text{slope})}$ was determined for each primer using standard curves generated from serial cDNA dilutions. Relative gene expression was calculated using the $2^{-\Delta CT}$ method, with untreated samples used as calibrators and GAPDH as the econtrol. Primers were purchased from Integrated DNA Technologies (IDT) (Supplementary Table 2).

Statistics

Data were analysed with Prism™ software (v9.3.1, GraphPad, Boston, USA) and were considered statistically significant when p-values were <0.05 (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). The Kolmogorov–Smirnov test was used to assess the distribution. For values that passed the normality test, parametric tests were applied: a paired t-test for two-group comparisons and a paired ANOVA test followed by a Tukey post hoc test for multiple comparisons. For values that did not pass the normality test, nonparametric tests were used. For paired data, the Wilcoxon signed-rank test (two groups) or the Friedman test (more than two groups), followed by Dunn’s post hoc test with appropriate correction for multiple comparisons, was applied. For unpaired data, the Kruskal–Wallis test followed by Dunn’s post hoc test was used for multiple group comparisons. Migration data were analyzed using linear mixed-effects models to account for repeated measures. Treatment condition, time, and their interaction were included as fixed effects, with patient as a random effect. Analyses were performed in R using the lme4 and lmerTest packages. Model assumptions were verified by inspection of residuals, including normality, homoscedasticity, and linearity over time. Effect sizes were calculated according to Cohen.

RESULTS

Localization and quantification of ER stress proteins in inflamed human and mouse synovial membranes

Following our previous proteomic analysis providing bulk protein expression [13], ER stress proteins were detected by IHC on synovial biopsies from the same patients. BiP, HYOU1, MANF, PDIA4, GANAB, HSP90B1, TXNDC5, DNAJB11, LMAN1, ERP29, CALR were detected, though TXNDC5 remained consistently low. In mildly inflamed tissues (Tak <4), expression restrained to the lining layer, while in highly inflamed samples (Tak $\geq$ 4), proteins extended into the sublining (Figure 1a). In the quantification, MANF, PDIA4, GANAB, TXNDC5, and CALR were significantly overexpressed in RA (Figure 1b). Differences for BiP, HYOU1, HSP90B1, DNAJB11, and LMAN1 were not statistically significant, likely due to overlapping Tak scores (Supplementary Figure 1). Differential expression exists in positive cells between non-inflammatory (NIN) and inflammatory (IN) conditions in OA and CPPA for MANF, PDIA4, HSP90B1, DNAJB11, ERP29 and CALR (Supplementary Figure 1).

Imaging mass cytometry, performed on the second cohort illustrates the same pattern of ER stress protein infiltration into the sublining as inflammation progresses (in NIN OA, IN OA and RA - Figure 2a). As shown in Figure 2a, their expression was restricted to the lining in Tak 0, observed predominantly in the sublining in Tak 4, and extended to the whole synovium in Tak 13. This is further supported by a semi-supervised clustering analysis expressed as fold change, calculated as the ratio of each chaperone's expression in a given region to its average across all the regions in the three biopsies. Four distinct clusters (regions 1 to 4, color-coded) emerged based on the mean expression of ER stress proteins. Column values represent the mean fold change across all chaperones, revealing a gradient from low expression in region 1 (blue) to high expression in region 4 (orange) (Figure 2b). In figure 2c, the low-expression blue region (or blue column) is predominantly located in the sublining independently of the inflammatory status. By contrast, the intermediate-expression yellow region (or beige column) is mainly present in the lining in both NIN and IN OA, while the highest-expression orange region (red column), of particular interest, infiltrates the sublining as inflammation advances. All the regions extend throughout the synovium in RA (Figure 2c).

IHC analysis in CIS mouse model confirmed increased expression of six chaperones (MANF, PDIA4, HSP90B1, DNAJB11, LMAN1 and CALR,) in inflamed synovium compared to controls (Supplementary Figure 2a-b). MANF, LMAN1, and DNAJB11 showed a positive correlation with pro-inflammatory cytokines (*Il1b* and/or *Il6*); PDIA4, HSP90B1, and CALR with the fibrotic marker *Acta2*; and MANF, HSP90B1, DNAJB11, LMAN1, and CALR with synovial thickness (Supplementary Figure 2c).

Targeting and localizing synovial cells of interest within the human inflamed synovial membranes

Based on the progression of expression and infiltration of ER stress proteins, we aimed to investigate which synovial cellular markers, among all the data obtained by the IMC™, exhibited similar patterns. Computational analysis identified potential cell subpopulations expressing these proteins, with 3 markers that stood out from our analysis: CD55 (lining FLS), CD34 (sublining FLS) and CD68 (MLS) (Figure 3a). Sublining FLS were distinguished from endothelial cells by the exclusion of CD31 marker ($CD34^{+}CD31^{-}$). Illustrated by red dots (high protein level expression), CD55 showed a strong expression in the lining layer of OA but completely disappeared in RA. In contrast, CD34, was primarily found in the sublining layer of inflamed OA but less in the lining as observed with NIN OA. CD34 was present throughout the synovial tissue in RA, which had lost its typical architecture. CD68 exhibited a similar expression pattern to CD55 but remained in RA tissues (Figure 3a). Both $CD3^{+}$, $CD4^{+}$ T cells and $CD20^{+}$ B cells were present within the lymphoid infiltrates in both NIN and IN OA, while in RA, these lymphocytes were more diffusely distributed throughout the synovial tissue (Figure 3b).

Co-expression of ER stress proteins with fibroblast-like synoviocytes

Based on our initial investigations of the localizations of ER stress proteins and synovial cells ($CD55^{+}$, $CD34^{+}CD31^{-}$, and $CD68^{+}$), we sought to determine, whether these ER stress proteins were co-expressed with one of these cell specific subpopulations. IMC™ approach aimed to identify potential interactions or relationships between these proteins and specific cell types within the synovial membrane. As ER stress proteins co-expressed with each other (Supplementary Figure 3), PDIA4 was selected for clarity to represent the entire set of chaperone proteins (Figure 4a). In the NIN OA, ER stress proteins are predominantly localized in the lining layer. In this compartment, they co-expressed with $CD55^{+}$ FLS and with $CD68^{+}$ MLS, while $CD34^{+}CD31^{-}$ FLS are nearly absent. In the IN OA, ER stress proteins infiltrate the

sublining and co-expressed with CD34⁺CD31⁻ FLS, while CD55 and CD68 expression remain confined to the lining (more abundant for CD55). In RA, the synovial architecture is disrupted. CD55 expression is lost, whereas CD34, CD68, and chaperones are widely distributed throughout the synovial tissue (Figure 4a). For lymphocyte markers (CD3, CD4, and CD20), both in NIN OA and IN OA, their expression was predominantly confined to lymphoid nodules, spatially distinct from the areas where ER stress proteins were detected. CD4 expression was also observed in the lining regardless of the inflammatory status, likely reflecting CD4 expression by macrophages themselves [282,283] (Figure 4a). In RA, these markers are widespread within the whole synovium, especially for CD3 and CD4 (Figure 4a).

The co-expression patterns were confirmed by a supervised clustering analysis of all ER stress proteins within each cellular marker population. This analysis identified two distinct clusters: i) low co-expression (low fold change) in the blue column representing the green region; and ii) high co-expression (high fold change) in the red column representing the blue region (Figure 4b). This latter region (blue region/ red column) was predominantly associated with CD55⁺ and CD68⁺ cells in the lining layer of NIN OA (Figure 4c). In contrast, under inflammatory conditions (Tak ≥ 4), most ER stress proteins shifted toward the sublining and co-expressed primarily with CD34⁺CD31⁻ fibroblasts, as illustrated by the spatial representation of supervised clustering analysis (Figure 4c).

10.1.1. Confirmation of ER stress protein co-expression with FLS on a larger cohort using immunofluorescence

These co-expression patterns were validated in an extended cohort of 25 OA patients using IF (Cohort 3- CHU Liège). First, co-expression of the ER stress protein PDIA4 with FLS markers was confirmed through the detection of their respective fluorescence signals (Figure 5a-d).

In NIN OA (Tak 3), there was a strong co-expression of ER stress proteins, represented by PDIA4, with CD55⁺ and CD68⁺ cells within a well-preserved lining layer (Figure 5a). This co-expression was still evident at an intermediate inflammatory grade (Tak 5) (Figure 5b). When inflammation worsens, the pattern shifts: at Tak score 6, a weak PDIA4 co-expression with CD55⁺ and CD68⁺ cells persists in the lining, but PDIA4 has already infiltrated the sublining and now co-expresses with CD34⁺CD31⁻ FLS (Figure 5c). At a higher inflammatory level (Tak 7), the lining is almost erased and the sublining is dominated by CD34⁺CD31⁻PDIA4⁺ FLS (Figure 5d).

Second, a quantitative phenotypic distribution analysis was performed on the lining and sublining, depending on the inflammatory gradient (Figure 5e-f). For CD55 (orange), in the lining (figure 5e), proportion of CD55⁺PDIA4⁺ cells was higher in NIN OA (Tak $\leq$ 3, 30%) and decreased in IN OA (Tak >6, 20%), while in the sublining (figure 5f), it remained low and stable (14% vs 15%). For CD68 (dark blue), the proportion of CD68⁺PDIA4⁺ cells increased with inflammation, from 17% to 28% in the lining and from 11% to 17% in the sublining. Of interest, for CD34⁺CD31⁻ (grey), the proportion of CD34⁺CD31⁻PDIA4⁺ cells markedly increased with inflammation, from 7% to 34% in the lining and from 9% to 31% in the sublining (Figures 5e-f). Among these, CD34⁺CD31⁻ fibroblasts represent the predominant PDIA4⁺ subpopulation in both the lining (34%) and sublining (31%) compartments under inflammatory conditions. Proportion of PDIA4⁻ cells (CD68⁺PDIA4⁻, CD55⁺PDIA4⁻, CD34⁺CD31⁻PDIA4⁻, CD55⁺CD34⁺CD31⁻PDIA4⁻ cells; green), in the lining and sublining progressively diminished with inflammation; in the sublining, it ranged from 64% in NIN OA to 34% in IN OA. These results indicate a redistribution of PDIA4 expression across cell types, with CD34⁺CD31⁻ fibroblasts emerging as the main contributors to the PDIA4⁺ pool under inflammatory conditions. These findings were validated using another ER stress protein, CALR, following the same experimental approach, including representative staining (Supplementary figure 4a-d) across increasing Tak scores and quantitative analyses in the lining (e) and sublining (f). Both qualitative and quantitative analyses confirmed that with increasing inflammation, the proportion of CD34⁺CD31⁻CALR⁺ cells increases, whereas CD55⁺CALR⁺ and CD68⁺CALR⁺ populations decrease (Supplementary Figure 4).

We then sought to determine whether CD55⁺ and CD34⁺ cells represent two distinct populations within the synovial membrane, or whether CD34⁺ cells might rather arise from a transition of CD55⁺ cells toward a CD34⁺ phenotype. Interestingly, we identified the presence of a CD55⁺CD34⁺CD31⁻ population (yellow) through co-staining (Figure 5e-f). Additionally, a negative correlation (Spearman, $r = -0.43$, P value: 0.03) was observed between CD55⁺PDIA4⁺ cells in the lining and CD34⁺CD31⁻PDIA4⁺ cells in the sublining across increasing Tak scores, whereas there was no correlation if these fibroblasts did not express this protein (Figure 5g-h, respectively). This suggests that PDIA4 may be involved in the progressive transition between the two fibroblast populations located in the lining and sublining layers.

ER stress, inflammation and pro-fibrotic conditions modulate chaperone expression and secretion in OA FLS through differential UPR pathway regulation

To investigate ER stress protein regulation in OA FLS, we first confirmed CD55 and CD34 expression by IF and WB (Supplementary Figure 5a and b, respectively). Both markers were present at baseline and remained unchanged upon ER stress (tunicamycin), or inflammatory (TNF- α) stimulation (Supplementary Figure 5b). FLS treated with tunicamycin for 1, 3, or 7 days showed significant induction of BiP, HYOU1, MANF, PDIA4, HSP90B1, LMAN1, and CALR (Figure 6a-b), while GANAB, TXNDC5, DNAJB11, and ERP29 were not modulated (quantification not shown). Most proteins (except BiP) were already detectable at baseline. We then assessed their secretion. BiP, PDIA4, HSP90B1, ERP29, and CALR were secreted after prolonged tunicamycin exposure (Figures 6c-d). While only PDIA4 was detectable in unstimulated supernatants, ERP29 secretion occurred exclusively at day 7. HYOU1, MANF, GANAB, TXNDC5, DNAJB11, and LMAN1 were not secreted under any condition (data not shown). Under inflammatory stimulation (TNF- α), BiP was overexpressed at day 1, while HYOU1, MANF, and PDIA4 increased at day 3 (Figures 6e-f). TGF- β stimulation led to elevated BiP at days 1 and 3, and increased HYOU1, MANF, and DNAJB11 expressions at day 3 (Figures 6g-h). Quantifications are not shown for proteins that were not modulated by TNF- α and/or TGF- β .

Altogether, five proteins—HYOU1, MANF, PDIA4, DNAJB11, and CALR—were consistently modulated by ER stress, inflammatory, or pro-fibrotic stimulation (Supplementary Table 3). They were therefore selected for further investigation as potential regulators of synovial fibroblast activation. To identify the signaling pathways involved in their regulation, cells were pretreated with selective inhibitors of the three canonical UPR branches—IRE1 α (4 μ 8c), PERK (PERK-IN-2), and ATF6 (Ceapin-A7) (Figure 7). Inhibitor efficacy was confirmed by reduced IRE1 α and EIF2 α phosphorylation, and decreased ATF6 cleavage (Figure 7). Accordingly, HYOU1 expression was regulated by both IRE1 α (Figure 7a-b) and ATF6 (figure 7e-f), and PDIA4 specifically by ATF6 (Figure 7e-f). PERK inhibition did not modulate any chaperone expression (Figure 7c-d). In line with this, inhibition of IRE1 α (Supplementary Figure 6a-b) and, to a lesser extent, inhibition of PERK (c-d) reduced α SMA expression, further supporting a role for UPR signaling in the regulation of pro-fibrotic pathways in OA FLS, whereas ATF6 inhibition had no effect (e-f).

PDIA4 drives proliferation and migration of OA FLS via TGF- β signaling

To assess proliferation and migration, FLS were transfected with siRNAs designed to silence the expression of the genes encoding these five proteins prior to stimulation with TGF- β for 3 days (Supplementary Figures 7a–d). Proliferation was significantly increased in control cells upon TGF- β stimulation (Figure 8a). This response was dependent on PDIA4, as PDIA4 silencing under TGF- β stimulation significantly reduced proliferation compared with control siRNA-treated cells (siCtrl + TGF- β vs. siPDIA4 + TGF- β , highlighted in bold). Of note, silencing HYOU1, PDIA4, DNAJB11, MANF or CALR did not affect cell viability (Figure 8b). These findings suggest that PDIA4 appears to be involved in proliferative processes within the synovium. This is supported by its co-expression with the proliferation marker Ki67 by IMC, with semi-supervised clustering identifying three co-expression levels (cyan: low, green: intermediate, dark blue: high) (Supplementary Figure 8a), and by spatial mapping revealing intermediate co-expression in the lining of NIN OA, strong sublining co-expression in IN OA, and a diffuse pattern in RA (Supplementary Figure 8b).

We next investigated whether PDIA4 also contributes to FLS migration. As expected, TGF- β induced migration in a time-dependent manner, with no significant effect at early time points and a progressive increase thereafter (Figure 8c). Importantly, PDIA4 silencing reduced TGF- β -induced migration at later stages compared to control conditions, indicating that PDIA4 may promote the pro-migratory response of OA FLS (Figure 8c).

DISCUSSION

ER stress has emerged as a key driver of synovitis, not only by amplifying inflammatory signaling [268] but also by promoting fibroblast proliferation [263], angiogenesis [286], and fibrotic remodeling [267] of the synovial tissue. Building on these insights and guided by our previous proteomic approach [13], we investigated in the current study the histological distribution of ER stress proteins in the inflamed human OA synovium. When inflammation was mild (Tak <4), these proteins were primarily localized in the lining layer whereas when the inflammation was severe (Tak $\geq$ 4), these proteins were mainly observed in the sublining layer. Using IMC, we confirmed this pattern of protein infiltration, as well as the corresponding spatial dynamics of fibroblast subsets, although performed on an exploratory cohort with a limited number of samples (n = 3). Specifically, under NIN conditions, CD55⁺ fibroblasts were mainly restricted to the lining layer. In contrast, inflammation was associated with an increased

presence of CD34⁺CD31⁻ FLS in the sublining layer. Besides, the distribution of CD68⁺ (MLS) cells was similar to that of CD55⁺ cells, but they remained detectable in RA, unlike these cells. These results are consistent with Culemann *et al* [162] identifying the presence of a palisade-like shape formed by MLS covering the lining of FLS and generating an internal immunological barrier at the synovial lining. This barrier restricts the inflammatory reaction by providing a tight-junction-mediated barrier that is typically played by epithelial cells in other tissues. Inflammation disrupts cell-to-cell contacts between MLS, thereby altering the structure of the lining layer [162]. RNA-seq data further suggest that FLS undergo a phenotypic switch during synovial inflammation [179]. Lining fibroblasts lose the expression of genes involved in maintaining cellular homeostasis and acquire a pro-inflammatory signature, similar to that of sublining fibroblasts, notably through the secretion of IL-6 [179–181,370]. We next sought to determine whether the observed patterns of FLS distribution and infiltration were reflected by the expression of the ER stress protein within these cell subsets (CD55, CD34, CD68) across inflammatory grades. The results were consistent with those obtained from a larger cohort using IF. As inflammation progressed, the co-expression of CD55⁺ FLS in the lining with PDIA4 (the reference ER stress protein) was lost. At the same time, CD34⁺CD31⁻ cells progressively acquired PDIA4 expression as this protein infiltrated the sublining. In other words, as inflammation progressed, the PDIA4⁻ cells in the sublining decreased progressively, giving way to an expanding CD34⁺CD31⁻PDIA4⁺ FLS population. The negative correlation between PDIA4-expressing CD55⁺ FLS in the lining and PDIA4-expressing CD34⁺ FLS in the sublining across increasing inflammatory grades, together with the presence of a CD55⁺CD34⁺CD31⁻ intermediate FLS subpopulation, points to a continuum of cellular states. This suggests that a gradual phenotypic transition driven by PDIA4 expression is more likely than a binary on/off switch in PDIA4⁺ cells. Interestingly, no correlation was observed between the two cellular subgroups when they did not express the protein. Consistently, the pro-migratory role of PDIA4 further supports a dynamic, non-binary process underlying fibroblast phenotypic changes. However, these experiments were performed in bulk OA FLS without isolation of CD55⁺ cells, precluding conclusions on whether this reflects a transition from CD55⁺ to CD34⁺ fibroblast subsets. An alternative approach would be to isolate CD55⁺ FLS and monitor whether their phenotype progressively shifts toward CD34⁺CD31⁻ cells. The key role of FLS in the expression of ER stress proteins aligns with previous data reporting the expression of MANF in FLS. However, this study did not distinguish between the different fibroblast subpopulations [290]. Finally, in our study, ER stress proteins were not expressed in lymphocytes.

We have also demonstrated that HYOU1 and PDIA4 are regulated by UPR pathways in OA FLS. Importantly, the link between UPR signaling and fibrotic processes has been well described in tumor and epithelial cells [338,339,371], and more recently in OA FLS [267]. Accordingly, we have shown that inhibition of IRE1 α and PERK reduces α -SMA expression, supporting a role in fibroblast-to-myofibroblast (FMT) transition. Taken together, these observations suggest that ER stress proteins may contribute to a phenotypic shift of OA FLS toward a proliferative, migratory and pro-fibrotic state, which we therefore sought to investigate under ER stress, pro-inflammatory or pro-fibrotic conditions.

The expression of CD55 or CD34 in FLS was first confirmed *in vitro*. However, their expression levels remained stable upon stimulation, suggesting that isolated stimuli are insufficient to induce a full phenotypic switch. The expression of studied chaperone proteins (except for BiP) was also observed in FLS without stimulation contributing to cell homeostasis. In contrast, their overexpression was highlighted under these conditions, leading to their secretion for some of them during prolonged ER stress. Interestingly, several chaperones are known to exhibit pro-inflammatory properties when they are exposed to the cell membrane or secreted outside, thereby favouring the production of inflammatory cytokines [273,287]. This suggests their involvement in triggering arthritis, particularly when they are released. Among these proteins, BiP [298], HSP90B1 [287], PDIA4 [372], ERP29 [373] and CALR [273] have already been described as secreted. *In vitro*, we demonstrated that human FLS overexpressed BiP, HYOU1, and MANF under ER stress, pro-inflammatory, and pro-fibrotic conditions (Supplementary Table 3). Additionally, PDIA4 and CALR were overexpressed and secreted *in vitro* by human FLS under prolonged ER stress. PDIA4 was also enhanced under pro-inflammatory conditions, whereas DNAJB11 was overexpressed under the pro-fibrotic process. Based on these findings, we selected the ER stress proteins HYOU1, MANF, PDIA4, DNAJB11 and CALR for further investigation into their role in driving proliferative processes in FLS. HYOU1 promotes the survival of neurons and phagocytes. It is also overexpressed in fibrotic kidneys, while it can be protective in certain fibrotic conditions [301,302,304–306]. HYOU1 is also linked to FMT and cytokine production in synovial fibroblasts [293] and has been reported to be overexpressed in injured synovium [307], supporting a role in synovial activation. MANF promotes neuronal survival, suppresses NF- κ B-mediated inflammation—particularly in fibroblasts—and reduces fibrosis in the liver [308,309,311–314]. It is increased in autoimmune diseases and involved in FLS from proliferative synovium [290,310]. PDIA4 and DNAJB11 are both linked to inflammation and fibrosis in various tissues, including the lungs, gut, adipose tissue, and skeletal muscle [316,318,319,374,375]. In particular, PDIA4 negatively regulates the onset of

arthritis [321], while DNAJB11 has been associated with autosomal-dominant polycystic kidney disease involving fibrosis [323,324], but remains unexplored in synovial pathology. Finally, CALR, which is overexpressed in RA synovium, contributes to FLS survival and angiogenesis [296,325–327] and has been identified as a stromal-derived antigen in RA synovial cells [326], highlighting it as a potential key target in synovitis.

HYOU1, MANF, PDIA4, DNAJB11, and CALR have previously been identified as having pro-proliferative effects in tumor cells, promoting cell survival and invasive behavior [347–351]. Synovitis is characterized by hyperplasia (cell proliferation) and inflammation that can lead to fibrosis in a chronic condition. This characteristic was investigated in FLS previously stimulated with TGF- β , in which the five ER stress proteins were silenced. Among them, PDIA4 was found to promote FLS proliferation in OA. This is the first study to describe the role of PDIA4 in the proliferation of OA FLS.

In conclusion, chaperones are detected in the lining layer of the synovial membrane under mild inflammatory conditions. As inflammation increases and the lining becomes disrupted, these ER stress proteins are expressed at higher levels by cells in the sublining. These proteins are expressed by CD55⁺ FLS and CD68⁺ MLS (NIN OA) in the lining, and by CD34⁺CD31⁻ FLS in the sublining (IN OA), and may be linked to the transition of their phenotype towards the deeper synovial layers. *In vitro* experiments using FLS further demonstrated that PDIA4 may be actively involved in proliferative and migratory processes, contributing to synovitis and OA disease progression, although its *in vivo* role in synovitis progression remains to be established.

This study provides the first spatial and functional characterization of ER stress proteins in synovitis. It links ER stress to fibroblast plasticity and reveals an association between chaperones and proliferative, migratory and pro-fibrotic processes in OA synovitis. Although restricted to OA-derived FLS, similar ER stress-driven mechanisms may also occur in RA and other inflammatory arthropathies.

STATEMENTS & DECLARATIONS

Acknowledgements

The authors thank the Immunohistochemistry and Imagery platforms of GIGA at the University of Liège for their contribution. The authors acknowledge the cytometry flow core facility Hyperion (Brest, France) for its technical assistance, as well as the European FEDER grant program Progos RU 000950 members of the Scientific Interest Group (GIS) Biogenouest.

Competing Interests

The authors declare that they have no financial or non-financial interests that are directly or indirectly related to the work submitted for publication.

Author contributions

Conceived and designed the experiments: dSD and GZ. Performed the experiments: GZ, NS, PZ, PS, HP, MN and CG. Analyzed the data: GZ, CP, HA, LG, CG and dSD. Provided joint tissues, FFPE synovial membranes and characterized synovial membrane inflammation: DC, TT, DP, BE and DP. Wrote the paper: GZ and dSD. Helped to conceive and draft the manuscript: GZ, SP, HA, DC, PC, PG, CG, RC and dSD. All authors read and approved the final manuscript.

Ethical approval and informed consent

All experiments were undertaken with patient material complying with the regulations and ethical guidelines of the University Hospital of Liège (CHU) and Catholic University of Louvain (UCLouvain) Belgium, and were approved by the CHU ethical committee (ref: 2017/147) and UCLouvain ethical committee (ref: 2013/24MAI/293) and the biobank research committee (BB190058) of the CHU of Liège. We certify that the study was conducted under the ethical standards outlined in the 1964 Declaration of Helsinki and its subsequent amendments, or with comparable ethical standards. Informed consent was obtained from all individual participants included in the study. All animal studies were conducted in compliance with European and Belgian guidelines for the care and use of laboratory animals, and experimentation procedures were approved by the local ethical committee (LA1610002; #14-1721 University of Liège, Liège, Belgium). The experiments comply with the current laws of Belgium in which they were performed. Investigators were not blinded to group allocation.

Funding

This study was supported by the “Fond d’Investissement pour la Recherche Scientifique” (FIRS, Centre Hospitalier Universitaire de Liège, Belgium), the Fondation Lejeune-Lechien, the Fondation Arthrose and a clinical research grant (Léon Frédéricq, Belgium) and the “Organisation Belge CAP48”. ZG is funded by the “Fonds National de la Recherche Scientifique” (FNRS).

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Supplementary information

The online version contains supplementary material.

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Figure legends

Figure 1. Localization of the 11 ER stress proteins in synovial membranes provided from patients with osteoarthritis (OA), chronic pyrophosphate arthropathy (CPPA) and rheumatoid arthritis (RA). a) Immunohistochemistry illustrations of the 11 ER stress proteins of interest provided from FFPE synovial membranes of OA ($n \geq 8$), CPPA ($n \geq 6$) and RA ($n \geq 5$) patients (Cohort 1); and b) their quantification (optical density values) obtained by QuPath. Highly positive cells have an $OD > 0.6$ (red spot), moderately positive $0.4 > OD > 0.6$ (orange spot), weakly positive $0.2 > OD > 0.4$ (yellow spot) and negative cells < 0.2 (blue spot). Data were analyzed with Prism 9 software (GraphPad). Graphs illustrate the median with a 95% confidence interval of the data. Statistical differences were considered for p-values < 0.05 (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Statistical analysis was performed using the nonparametric unpaired Kruskal- Wallis test followed by Dunn's post hoc test for multiple comparisons. Each point represents one biopsy (one patient), and n indicates the number of independent biopsies. ER: endoplasmic reticulum; NIN: non-inflammatory; IN: inflammatory and OD = optical density.

Figure 2. Spatial distribution and clustering of ER stress protein expression in synovial tissue. a) Representative multiparametric imaging mass cytometry (IMC) of synovial sections from patients with OA and RA (Cohort 2, $n=3$) showing localization of ER stress proteins. One NIN, one IN OA and one RA are selected across the 6 biopsies to represent the inflammatory continuum. b) Semi-supervised clustering of the 11 ER stress proteins reveals four distinct tissue regions based on protein expression patterns. c) Map of the semi-clustering by IMC of ER stress proteins in the same biopsies, represented by the four code-colored regions. The region 4 (orange) is defined by high expression level of ER stress proteins. ER: endoplasmic reticulum; NIN: non-inflammatory; IN: inflammatory; OA: osteoarthritis; RA: rheumatoid arthritis

Figure 3. Spatial representation of supervised clustering analysis of a) synovial fibroblast ($CD55^+$ and $CD34^+$) and macrophage ($CD68^+$) subsets; and b) T ($CD3^+$ and $CD4^+$) and B ($CD20^+$) lymphocytes in non-inflammatory OA, inflammatory OA, and RA. Red and blue dots respectively indicate areas with high or no expression of fibroblast, macrophage, T or B cell markers, as defined by the clustering algorithm. NIN: non-inflammatory; IN: inflammatory; OA: osteoarthritis; RA: rheumatoid arthritis

Figure. 4. Co-expression of PDIA4 with FLS in the synovial membrane. a) Multiparametric imaging mass cytometry (Cohort 2, n=3) of PDIA4, FLS/MLS and lymphocyte markers. White squares enclose marker infiltration. b) Supervised clustering analysis of ER stress protein expression within CD55⁺, CD68⁺, and CD34⁺CD31⁻ cell populations. c) Spatial representation of supervised clustering analysis of ER stress protein co-expression with synovial fibroblast and macrophage subsets in NI OA, IN OA, and RA. Blue dots indicate areas with high co-expression of ER stress proteins with the cell marker, as defined by the clustering algorithm, while green and white areas correspond to regions with lower co-expression. ER: endoplasmic reticulum; FLS: fibroblast-like synoviocytes; MLS: macrophage-like synoviocytes; NIN: non-inflammatory; IN: inflammatory; OA: osteoarthritis; RA: rheumatoid arthritis

Figure 5. Validation of ER stress protein co-expression with FLS in an extended OA cohort (Cohort 3, n=25) and their transitional phenotypic shift). Immunofluorescence analysis of PDIA4 (white), CD55⁺ (red), CD68⁺ (green) CD34⁺ (yellow), CD31⁺ (pink) and DAPI (blue) in a) non inflamed (Tak 3), b) intermediate grade (Tak 5), c) inflamed (Tak 6) and d) inflamed (Tak 7) OA synovial samples. White arrows show expression of PDIA4 within CD55⁺, CD68⁺ or CD34⁺CD31⁻ cells. e-f) Panels showing the proportion of CD68⁺, CD55⁺, CD34⁺CD31⁻, or CD55⁺CD34⁺CD31⁻ cells expressing PDIA4 among total CD68⁺, CD55⁺, CD34⁺CD31⁻ or CD55⁺CD34⁺CD31⁻ cells in the lining (e) and sublining (f), stratified by Tak score. g-h) Spearman analysis representing the correlation between CD55⁺ FLS in the lining and CD34⁺CD31⁻ FLS in the sublining, either expressing PDIA4 (g) or not expressing PDIA4 (h), with increasing Tak scores. A linear regression illustrates this inverse relationship. n = number of independent biopsies (one biopsy per patient). ER: endoplasmic reticulum; FLS: fibroblast-like synoviocytes; OA: osteoarthritis; NIN: non-inflammatory; IN: inflammatory.

Figure 6. Modulation of ER stress proteins after induction of ER stress, pro-inflammatory and pro-fibrotic process. Illustrative Western blot images depicting 11 ER stress proteins intracellular expression following 1, 3 and 7 days of a) Tm (1µg/mL), e) TNF-α (10 ng/mL), or g) TGF-β (10 ng/mL) stimulation on FLS and their respective semi-quantifications b) f) h). HSP90 or GAPDH expression were used as loading controls. Protein expression and secretion were assessed in parallel by analyzing both intracellular extracts (a–b) and matched extracellular supernatants following tunicamycin treatment (c–d). Ponceau S staining served as a protein-loading control. n≥6. Statistical analysis was performed using the nonparametric paired Friedman test followed by Dunn's post hoc test for multiple comparisons. Only

statistically significant quantifications are illustrated. Statistical differences were considered for p-values < 0.05 (*p<0.05, **p<0.01, ***p<0.001). Data are presented as median with 95% CI. Each point represents one measurement obtained from an individual patient under a specific stimulation condition. *n* indicates the number of independent patients. NT: non-treated; D:day. ER: endoplasmic reticulum; Tm: tunicamycin; TNF- α : tumor necrosis factor- α ; TGF- β : transforming-growth factor- β

Figure 7. UPR-dependent regulation of ER stress proteins in OA FLS. To investigate the regulatory pathways involved in the modulation of the five ER stress proteins, cells were pretreated with selective inhibitors of IRE1 α (4 μ 8c), PERK (PERK-IN-2) or ATF6 (Ceapin-A7) prior to a 1-day tunicamycin stimulation. Pathway inhibition was assessed by monitoring p-IRE1 α , EIF2 α phosphorylation or cleaved ATF6 (~50 kDa). a, c, e) Representative Western blots and b, d, f) their corresponding quantifications for IRE1 α (4 μ 8c), PERK (PERK-IN-2), and ATF6 (Ceapin-A7) inhibition, respectively. Statistical analysis was performed with the nonparametric paired Friedman test followed by Dunn's post hoc test for multiple comparisons. Statistical differences were considered for p-values < 0.05 (*p<0.05, **p<0.01, ***p<0.001). Data are presented as median with 95% CI; each point represents one patient. *n* indicates the number of independent patients. UPR: unfolded protein response; OA: osteoarthritis; FLS: fibroblast-like synoviocytes.

Figure 8. Effect of ER stress protein silencing on FLS proliferation, viability and migration.

a) FLS were transfected with siRNAs targeting HYOU1, MANF, PDIA4, DNAJB11, or CALR, then stimulated with TGF- β (10 ng/mL) for 3 days. Proliferation was measured by BrdU incorporation, and b) viability by MTS assay. Data are presented as median with 95% CI; each point represents one patient. Statistical significance was assessed using the nonparametric paired Friedman test followed by Dunn's post hoc test for multiple comparisons. *n* indicates the number of independent patients. c) Real-time migration of OA FLS monitored using the IncuCyte system. Cells were transfected with control siRNA (siCtrl) or siRNA targeting PDIA4 (siPDIA4), with or without a 3-day TGF- β stimulation. Migration was quantified as relative wound density (%) at the indicated time points (hours). Migration data were analyzed using linear mixed-effects models with treatment, time, and their interaction as fixed effects and patient as a random effect. Statistical differences were considered for p-values <0.05: *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001); FLS: fibroblast-like synoviocytes; Ctrl:

controls. ER: endoplasmic reticulum; TGF- β : transforming-growth factor- β ; siRNA: small interfering; BrdU: bromodésoxyuridine.

Tables

Supplementary Table 1

a.

	Cohort 1				Cohort 2		Cohort 3
n (total)	24				3		25
Methods	IHC				IMC		IF
Pathologies	OA	CPPA	RA		OA	RA	OA
P value				P value			
n	9	7	8		2	1	25
Gender (W/M)	8/1	5/2	5/3	0.44	1/1	1/0	21/4
Age [mean (+/- SD)] year	57.4 (+/-14.1)	64.6 (+/-8.5)	55.75 (+/-16.3)	0.3	78 (+/-5.6)	58	65 (+/-14.3)
BMI [mean (+/- SD)] kg/m2	30.3 (+/-7.3)	26.5 (+/-4.3)	25.2 (+/-6)	0.4	28.7 (+/-6.7)	23.83	28.9 (+/- 5.3)
Anti-ACPA	/	/	4/8	/	/	0	/
Rheumatoid factor	/	/	3/8	/	/	0	/
Tak score [mean (+/- SD)]	4.2 (+/-1.8)	5.7 (+/-2.1)	12.2 (+/-1.5)	0.0003	2 (+/-2.8)	13	4.3 (+/-2)

b.

Cohort	Pathology	Histological scores				Tak score
		Hs	Ly	Pl	Po	
1	OA	1	1	0	0	2
		2	1	0	0	3
		2	1	0	0	3
		2	1	0	0	3
		2	1	0	0	3
		3	2	0	0	5
		3	1	2	0	6
		3	2	1	0	6
		4	2	1	0	7
	CPPA	3	1	0	0	4
		3	1	0	0	4
		2	1	2	0	5
		3	2	0	0	5
		4	2	0	0	6
		2	3	1	0	6
	RA	4	3	1	2	10
		2	4	2	3	11
		4	3	1	2	11
		4	3	3	1	11
		4	3	4	0	11
		4	4	4	0	12
		3	4	4	3	14
		4	4	4	2	14
		4	4	4	2	14
2	OA	0	0	0	0	0
		1	2	1	0	4
	RA	2	4	4	3	13
3	OA	1	1	0	0	2
		0	2	0	0	2
		0	2	0	0	2
		1	1	0	0	2
		2	1	0	0	3
		2	1	0	0	3
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		2	1	2	0	5
		3	2	0	0	5
		2	3	0	0	5
		3	2	0	0	5
		3	2	1	0	6
		3	1	2	0	6
		3	2	1	0	6
		4	2	1	0	7
		4	3	1	0	8
		4	3	3	0	10

Supplementary Table 1. Description of the three cohorts: a) Demographic, clinical and biologic data. The P values were calculated using an unpaired Kruskal–Wallis test followed by Dunn’s multiple comparisons post hoc test; b) detailed Tak score for the three cohorts. W: women; M: men; SD: standard deviation; BMI: body mass index; ACPAs: anti-citrullinated protein antibody; IHC: immunohistochemistry; IF: immunofluorescence; IMC (imaging mass cytometry); MFI: mean fluorescence intensity; OA: osteoarthritis; CPPA: chronic pyrophosphate arthropathy; RA: rheumatoid arthritis; Hs: hyperplasy; Ly: lymphocytes; Pl: plasmocytes; Po: Polymorphonuclear leukocytes

Supplementary Table 2

Gene ID	Protein name	IHC		CIS (mouse)		IMC		IF (FFPE tissue)		Primary FLS		WB
		Cohort 1		Isotope	Reference	Clone	Isotope	Cell/protein type	Reference	Reference		
		Reference										
<i>HSP45</i>	Binding immunoglobulin protein (BiP)	3177, Cell Signaling		X	ab236050, Abcam	EPR4041(2)	160Gd	ER stress protein			3177, Cell signaling	
<i>HYOU1</i>	Hypoxia up-regulated protein 1 (GRP170)	ab134944, Abcam			MA5-29334, Thermo Fisher	201	148Nd	ER stress protein			ab134944, Abcam	
<i>MANF</i>	Mesencephalic astrocyte-derived neurotrophic factor	ab67271, Abcam		X	ab67271, Abcam	Rabbit Polyclonal	172Yb	ER stress protein			ab67271, Abcam	
<i>PDLA4</i>	Protein disulfide-isomerase A4	ab155800, Abcam		X	ab251013, Abcam	EPR14824(B)	168Er	ER stress protein			ab155800, Abcam	
<i>GANAB</i>	α -subunit of glucosidase II	ab176349, Abcam			ab249921, Abcam	EPR12376	169Tm	ER stress protein			ab176349, Abcam	
<i>HSP90B1</i>	Heat shock protein 90 beta family member 1	20292, Cell Signaling		X	ab256312, Abcam	EPR22847-50	146Nd	ER stress protein			20292, Cell signaling	
<i>TXNDC5</i>	Thioredoxin domain-containing protein 5	CF507281, Origen			CF507281, Fisher Scientific	OT12E6	154Sm	ER stress protein			CF507281, Origen	
<i>DNAJB11</i>	DnaJ homolog subfamily B member 11	ab224082, Abcam		X	PA5-117915, Thermo Fisher	Rabbit Polyclonal	145Nd	ER stress protein			ab224082, Abcam	
<i>LMAN1</i>	Lectin mannose-binding 1	ab125006, Abcam		X	ab248096, Abcam	EPR6979	175Lu	ER stress protein			ab125006, Abcam	
<i>ERP29</i>	Endoplasmic reticulum resident protein 29	ab176373, Abcam			ab249928, Abcam	EPR12499(B)	150Nd	ER stress protein			ab176373, Abcam	
<i>CALR</i>	Calreticulin	ab92516, Abcam		X	ab271865, Abcam	EPR3924	143Nd	ER stress protein			ab92516, Abcam	
<i>CD34</i>	Hematopoietic progenitor cell antigen CD34				ab198395, Abcam	EP373Y	139La	Sublining fibroblast				
<i>CD55</i>	Complement decay-accelerating factor				ab230797, Abcam	EP6869	149Nd	Lining fibroblasts			3569, Cell Signaling	
<i>CD31</i>	Platelet endothelial cell adhesion molecule				3151025D, Standard BioTools	EPR3094	151Eu	Endothelial cells			ab133684, Abcam	
<i>CD4</i>	T-cell surface glycoprotein CD4				3156033D, Standard BioTools	EP6855	156Gd	T helper			98025, Cell Signaling	
<i>CD68</i>	Macrosialin				3159035D, Standard BioTools	KP1	159Tb	Macrophages			sc20060, Santa Cruz	
<i>CD20</i>	B-lymphocyte antigen CD20				3161029D, Standard BioTools	H1	161Dy	B cells				
<i>CD3</i>	T-cell surface glycoprotein CD3 gamma chain				3170019D, Standard BioTools	Polyclonal, C-Terminal	170Er	T cells				
<i>MKI67</i>	Ki-67				ab237863, Abcam	MK-67/2462	142Nd	Proliferation				

Gene ID	Forward primer (5'-3')	Reverse primer (5'-3')
<i>HYOU1</i>	GCT TCC TTA GTC TTC ACC ATC T	GAT ATT AAC ACC ACT GCC CAG A
<i>MANF</i>	GCT TCT CAC AGA TCT TCT CCA C	CAA GAG GCA AAG AGA ATC GGT
<i>PDLA4</i>	TCA ACA CAA GCG TGA CTT CT	GGC AGG CTG TAG ACT ACG A
<i>DNAJB11</i>	TCG TAC TGT TGC CTT TTC TCA C	AAC CCT GAT GAT CCA CAA GC
<i>CALR</i>	TTG GTG ATG AGG AAG TTG TCA	CCG ATC GCA GTA TCT ATG CCT A

Supplementary Table 2. List of antibodies and associated details used across experimental techniques including IHC, IMC, IF and WB on synovial tissue from Cohorts 1, 2, and 3, the CIS mouse model, and primary FLS; and list of primers used for qPCR. ID: identity; IHC: immunohistochemistry; IMC: imaging mass cytometry; IF: immunofluorescence; FFPE: formalin-fixed paraffin-embedded; WB: western blot; CIS: collagenase-induced synovitis; FLS: fibroblast-like synoviocytes

Supplementary Table 3

	IHC		WB			
	Cohort 1	CIS (mouse)	Primary FLS			
			ER stress (Tm)		Fibrosis (TGF- β)	Inflammation (TNF- α)
	Tissue	Tissue	Intracellular	Extracellular	Intracellular	Intracellular
BiP			X	X	X	X
HYOU1			X		X	X
MANF	X	X	X		X	X
PDIA4	X	X	X	X		X
GANAB	X					
HSP90B1		X	X	X		
TXNDC5	X					
DNAJB11		X			X	
LMAN1		X	X			
ERP29				X		
CALR	X	X	X	X		

Supplementary Table 3: Summary table of overexpressed ER stress proteins across experimental techniques including IHC and WB on synovial tissue from cohort 1, the CIS mouse model and primary FLS. Each cross represents the overexpression or secretion of the protein of interest in synovial tissues or in cells under ER stress (Tm), profibrotic (TGF- β), and proinflammatory (TNF- α) stimuli.

Figures

Figure 1

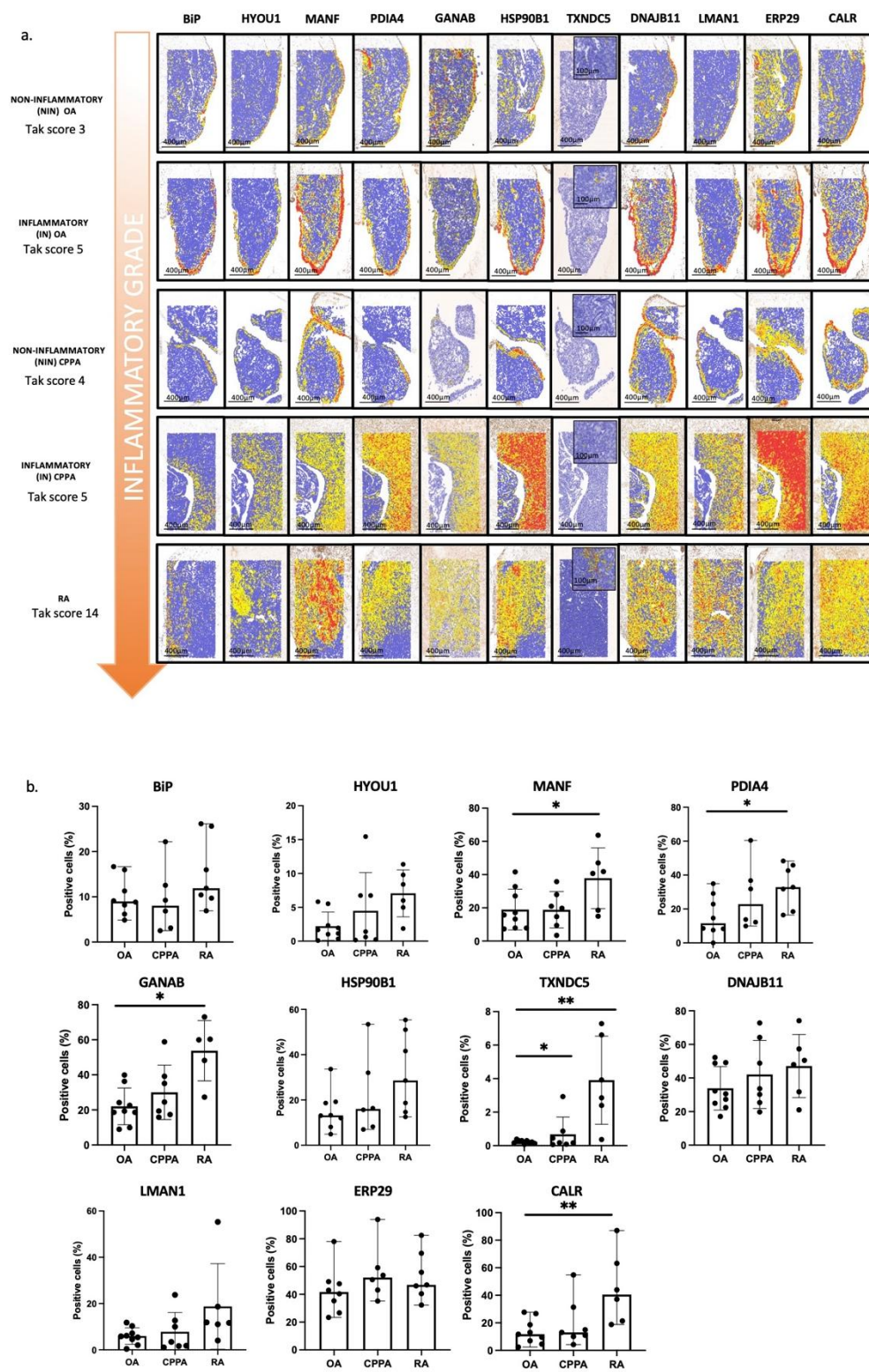


Figure 2

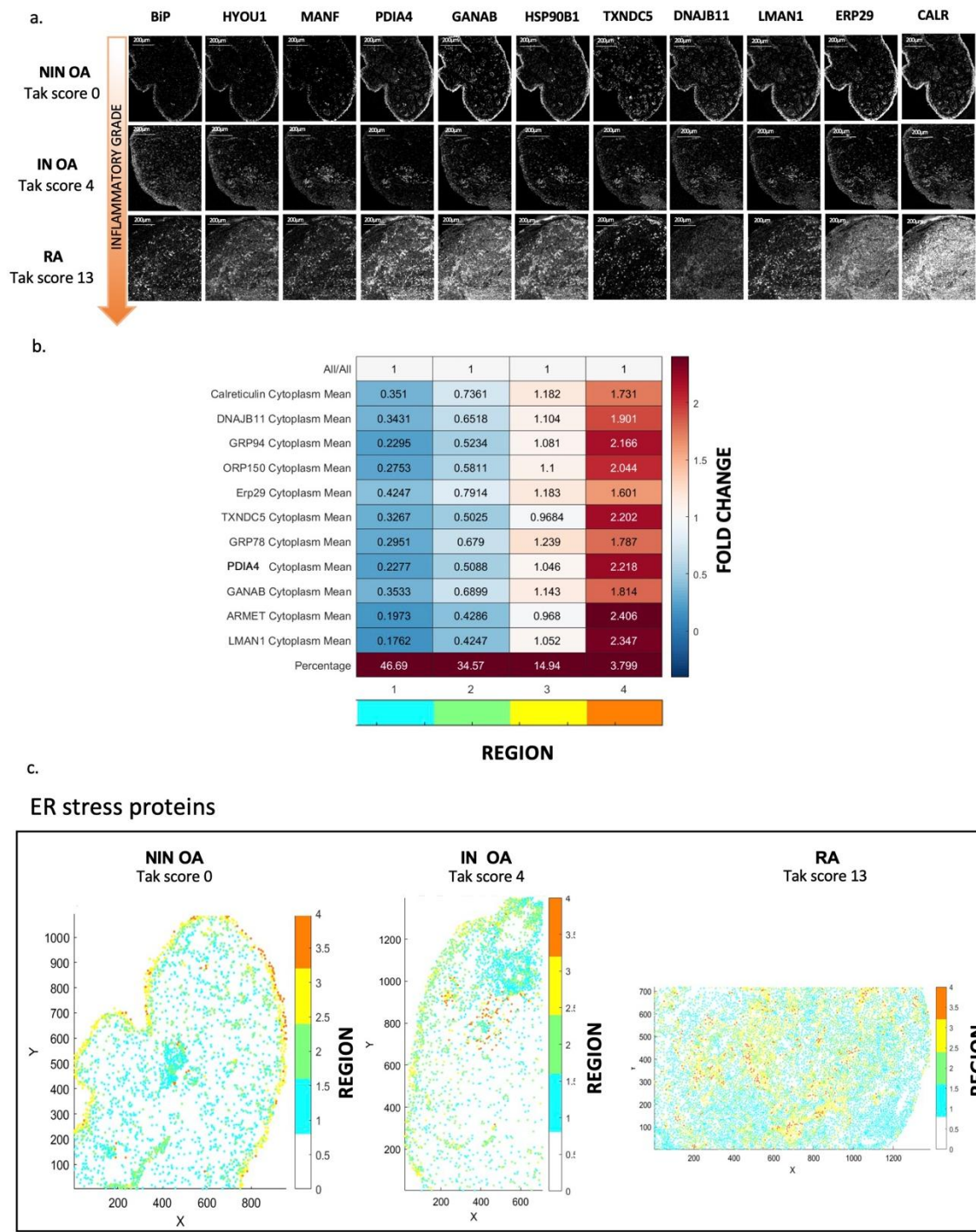
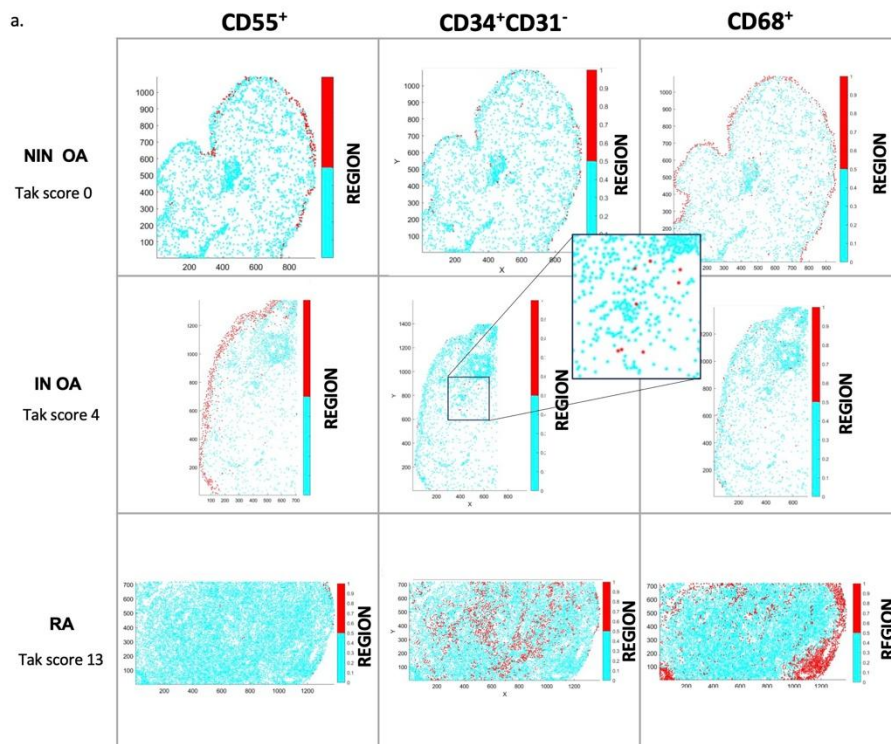


Figure 3

FLS/MLS markers



Lymphocytes markers

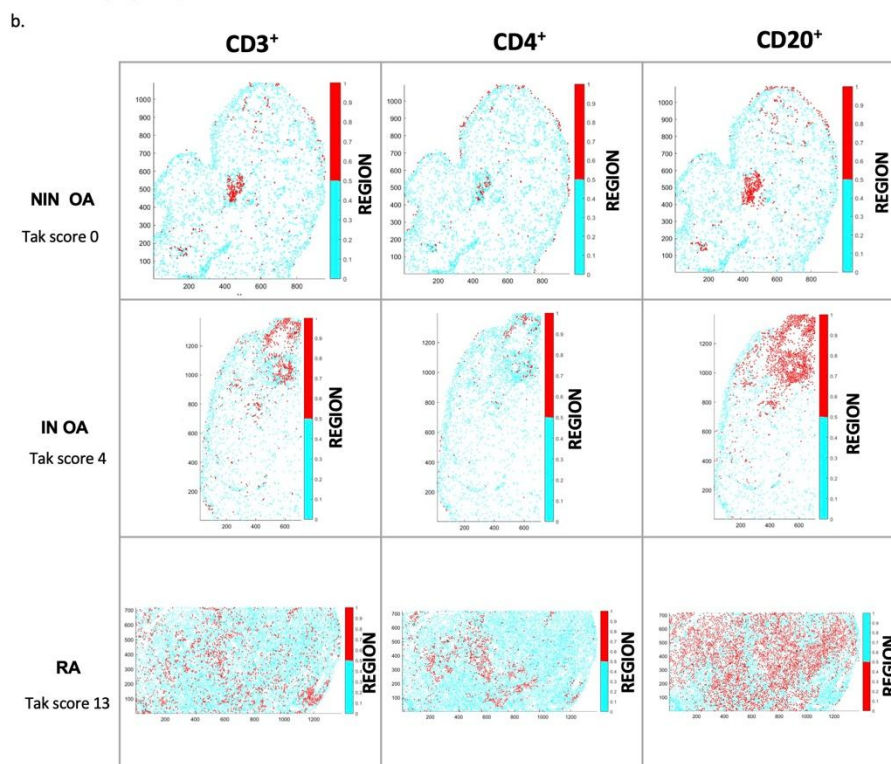


Figure 4

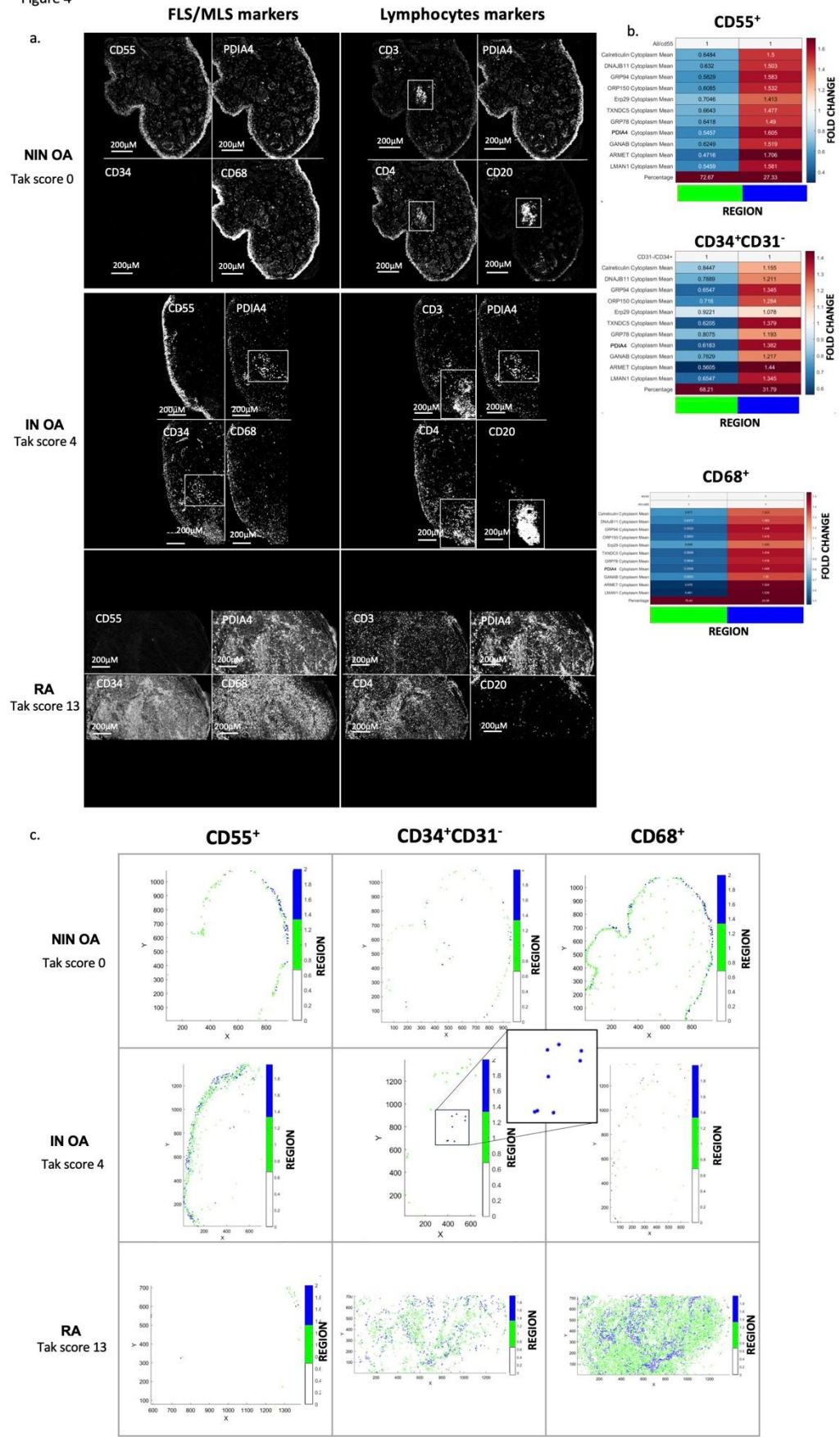
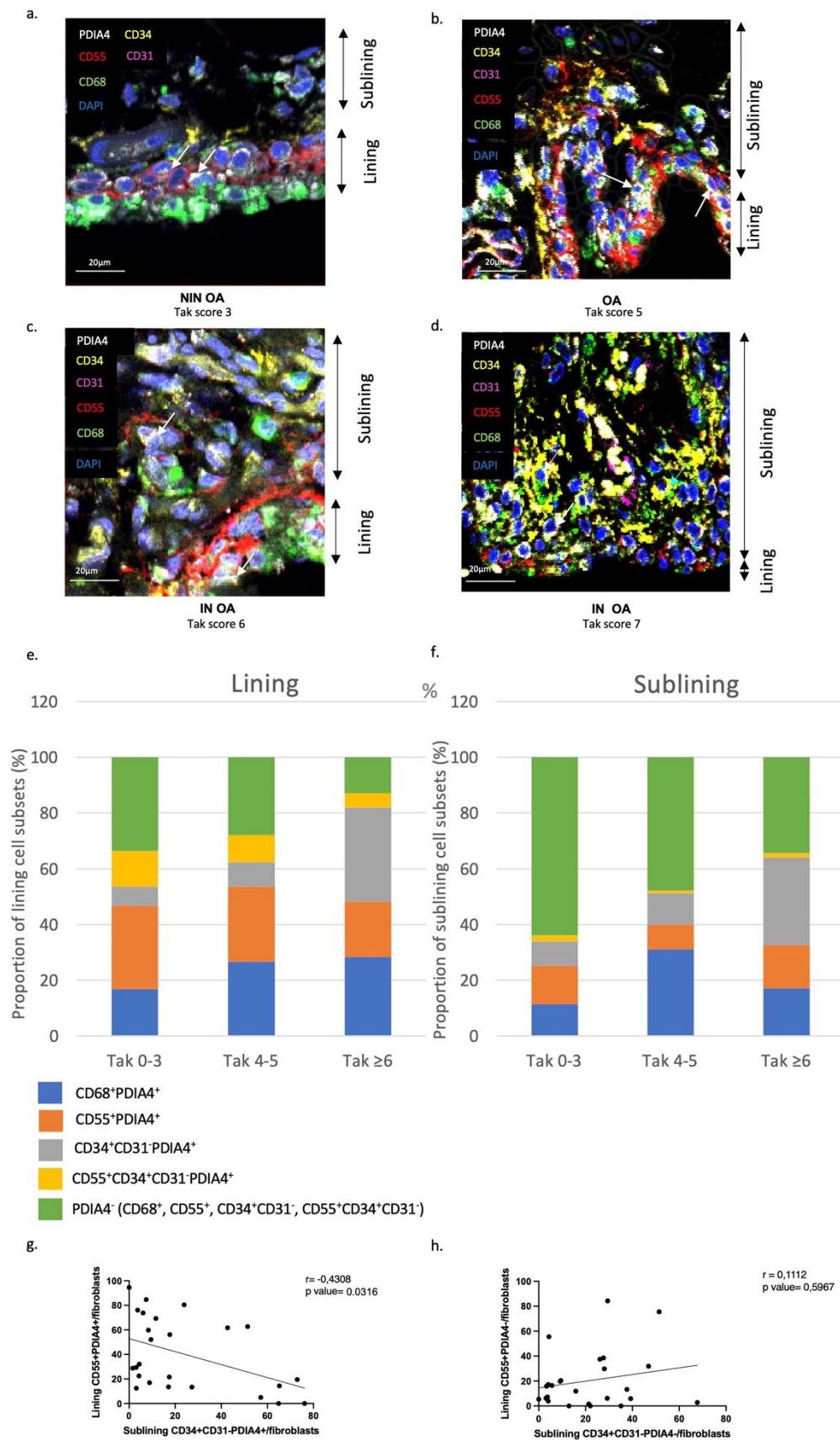


Figure 5



ER stress (Tunicamycin)	
1	2
3	4
5	6
7	8
9	10
11	12
13	14
15	16
17	18
19	20
21	22
23	24
25	26
27	28
29	30
31	32
33	34
35	36
37	38
39	40
41	42
43	44
45	46
47	48
49	50
51	52
53	54
55	56
57	58
59	60
61	62
63	64
65	66
67	68
69	70
71	72
73	74
75	76
77	78
79	80
81	82
83	84
85	86
87	88
89	90
91	92
93	94
95	96
97	98
99	100



Figure 7

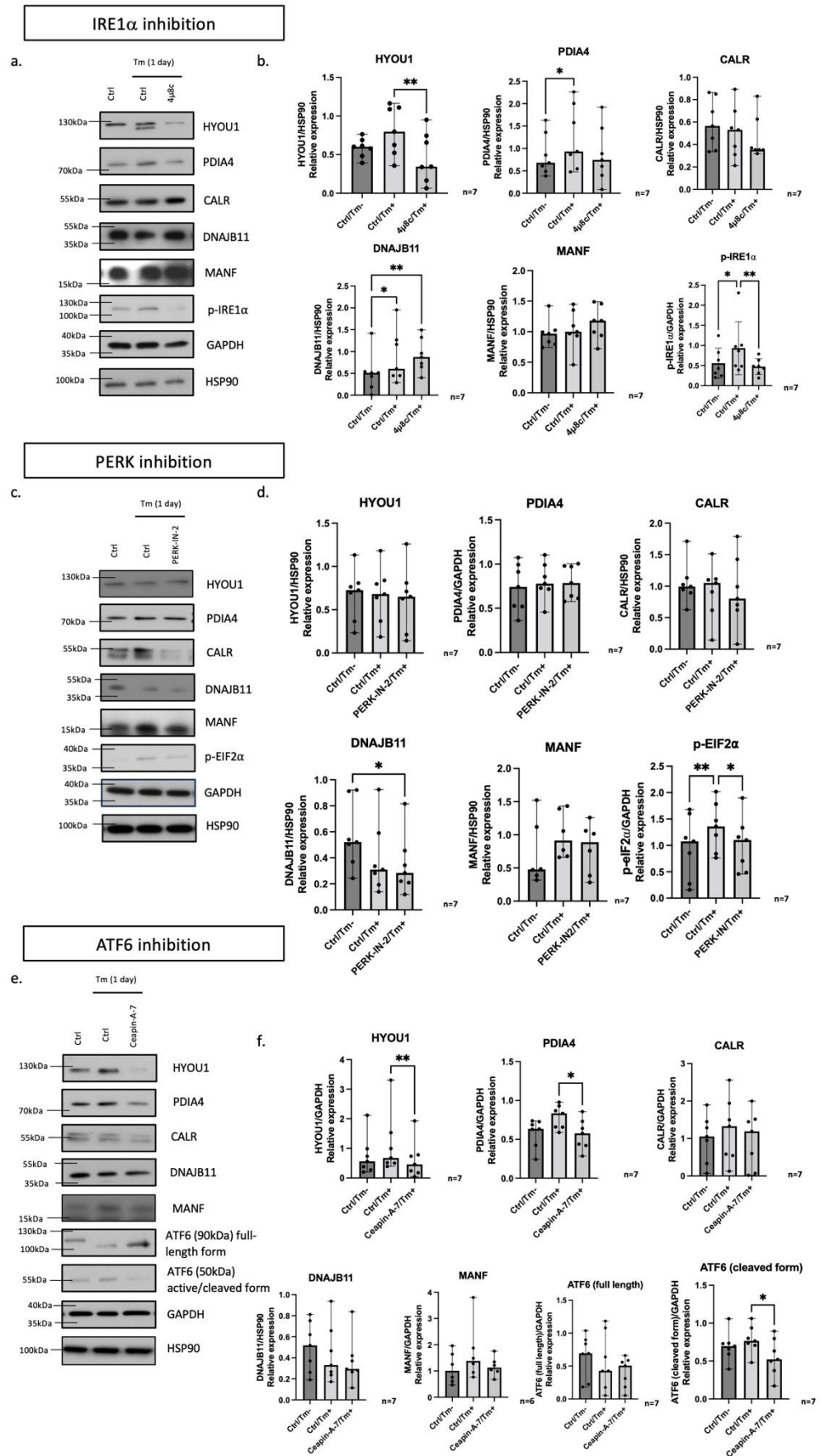
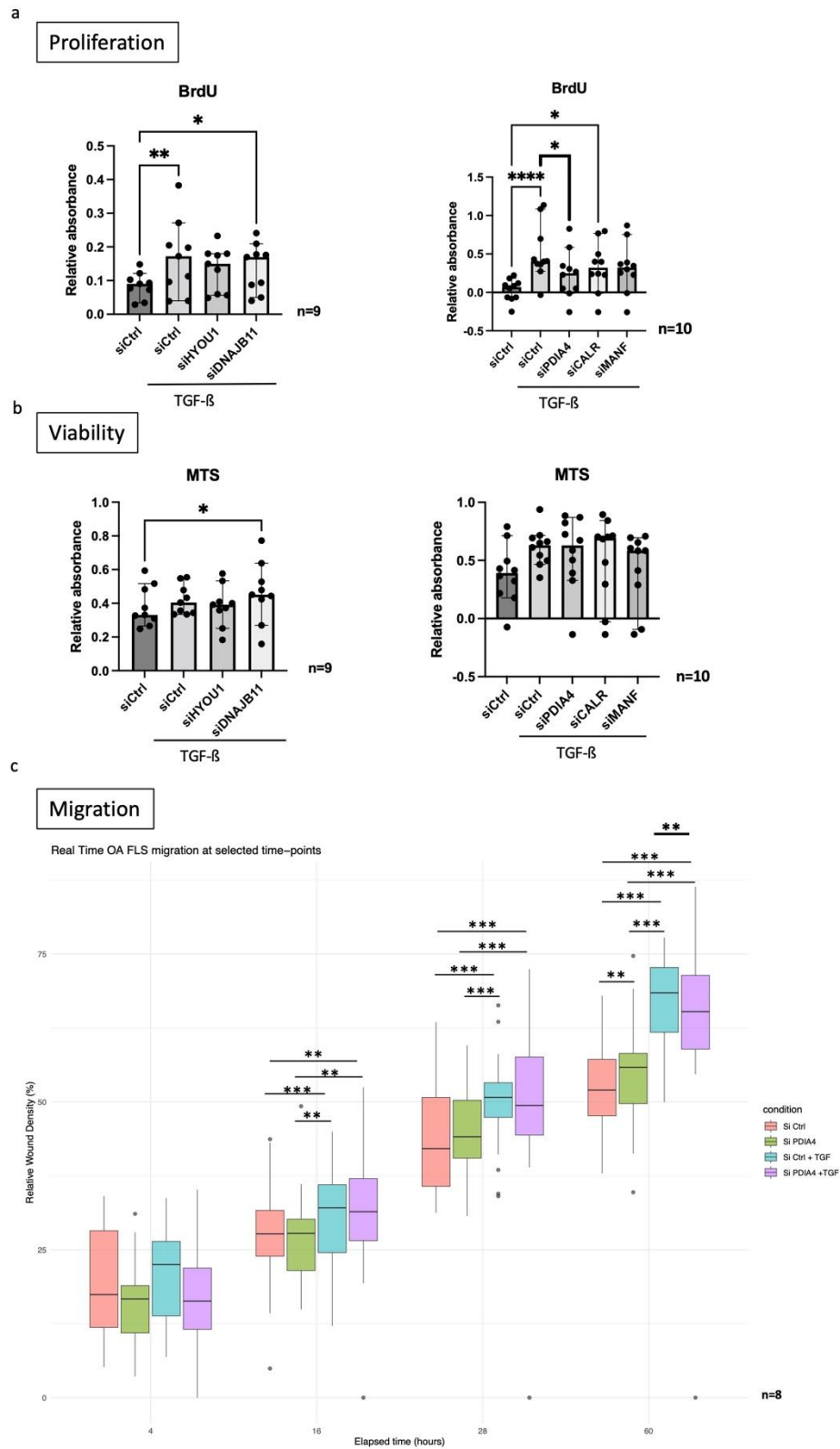
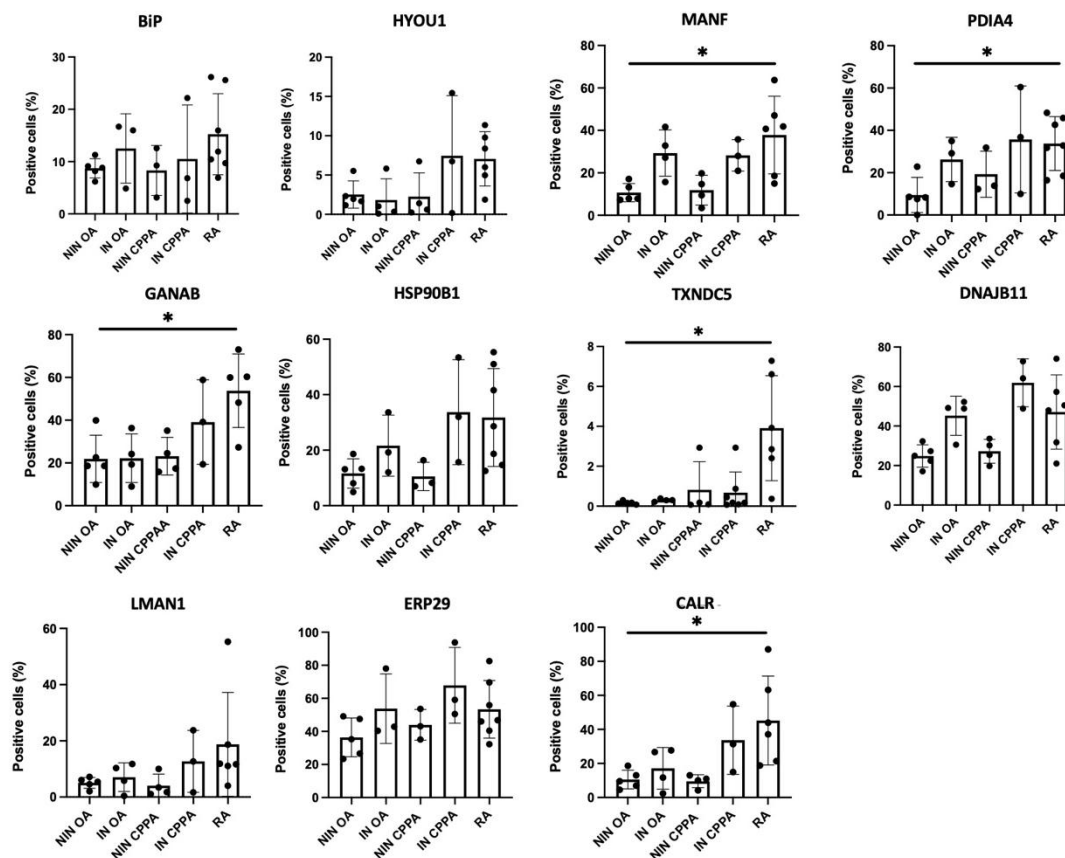


Figure 8

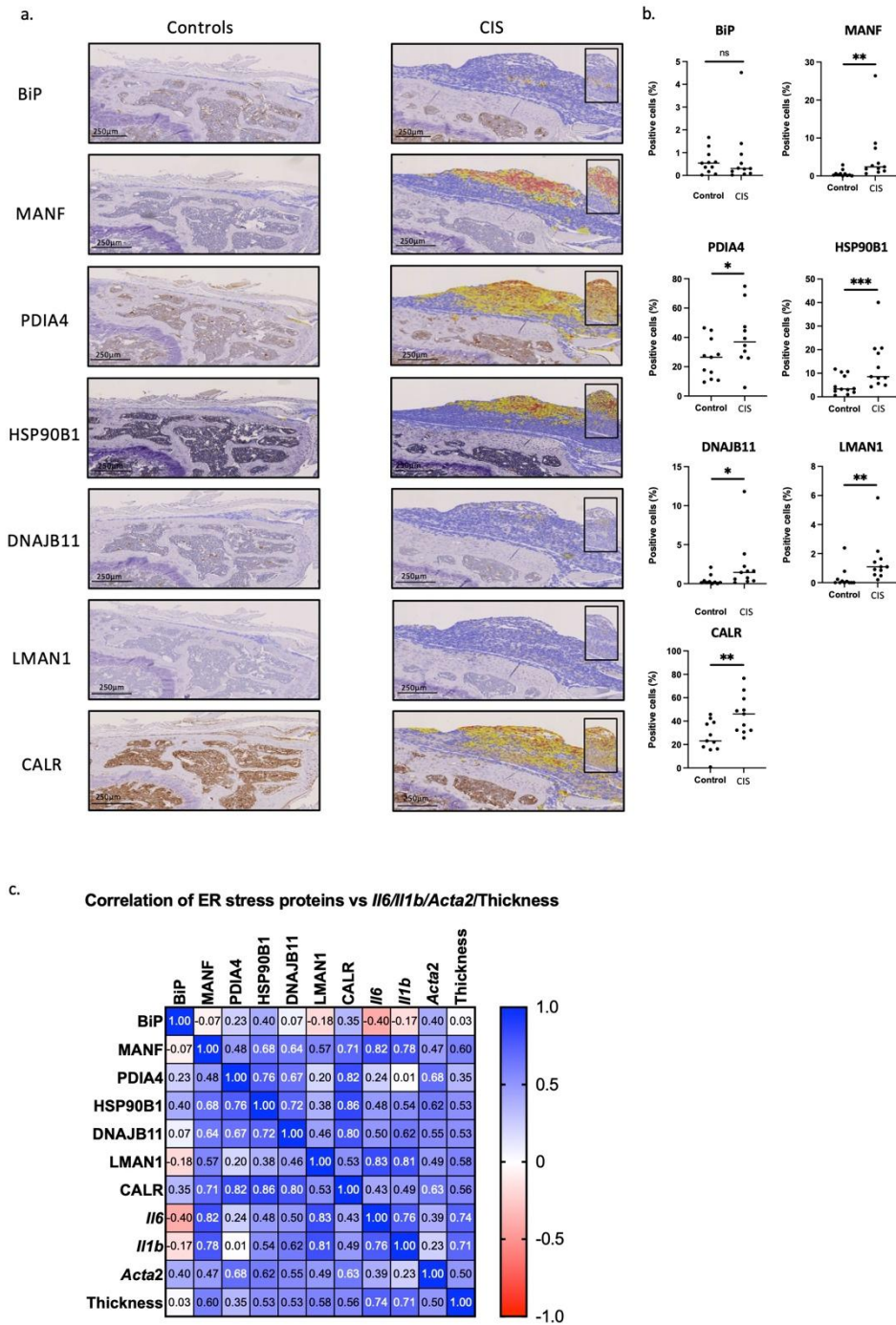


Supplementary Figure 1



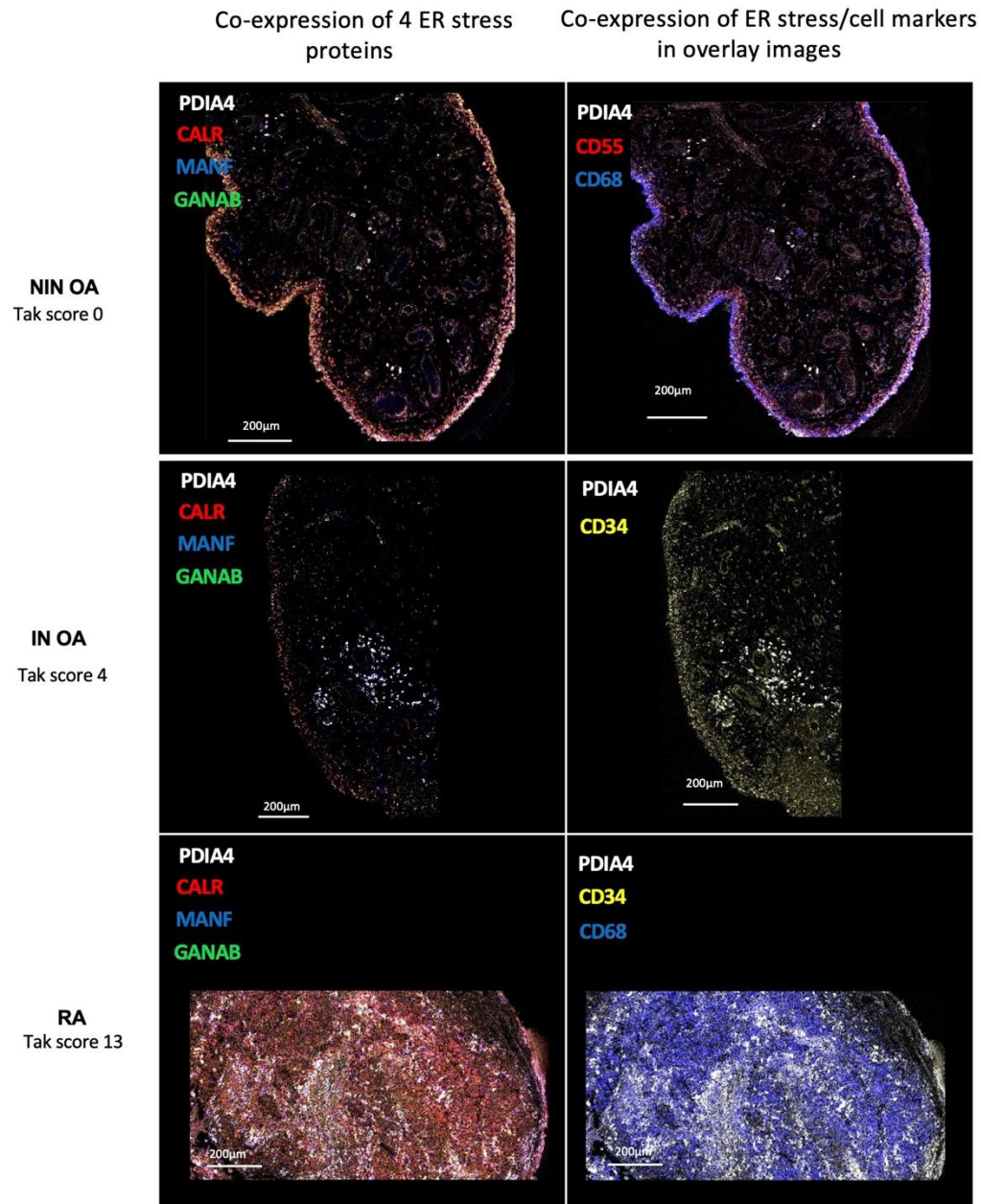
Supplementary Figure 1. Expression levels of the 11 ER stress proteins in synovial membranes provided from patient with (non)-inflammatory osteoarthritis (OA), (non)-inflammatory chronic pyrophosphate arthropathy (CPPA) and rheumatoid arthritis (RA). Quantification obtained by QuPath for the 11 ER stress proteins of interest provided from FFPE synovial membranes of non-inflammatory osteoarthritis (NIN OA), inflammatory osteoarthritis (IN OA), non-inflammatory chronic pyrophosphate arthropathy (NIN CPPA), inflammatory chronic pyrophosphate arthropathy (IN CPPA) and rheumatoid arthritis RA patients. Data were analyzed with Prism 9 software (GraphPad). Graphs illustrate the median with a 95% confidence interval of the data. Statistical differences were considered for p-values < 0.05, *p<0.05, **p<0.01, ***p<0.001. The nonparametric unpaired Kruskal-Wallis test followed by Dunn's post hoc test for multiple comparisons was applied on our data. Each point represents one biopsy (one patient). NIN OA (n≥5), IN OA (n≥3), NIN CPPA (n≥3), IN CPPA (n≥3), RA (n≥5). NIN= non inflammatory; IN= inflammatory.

Supplementary Figure 2



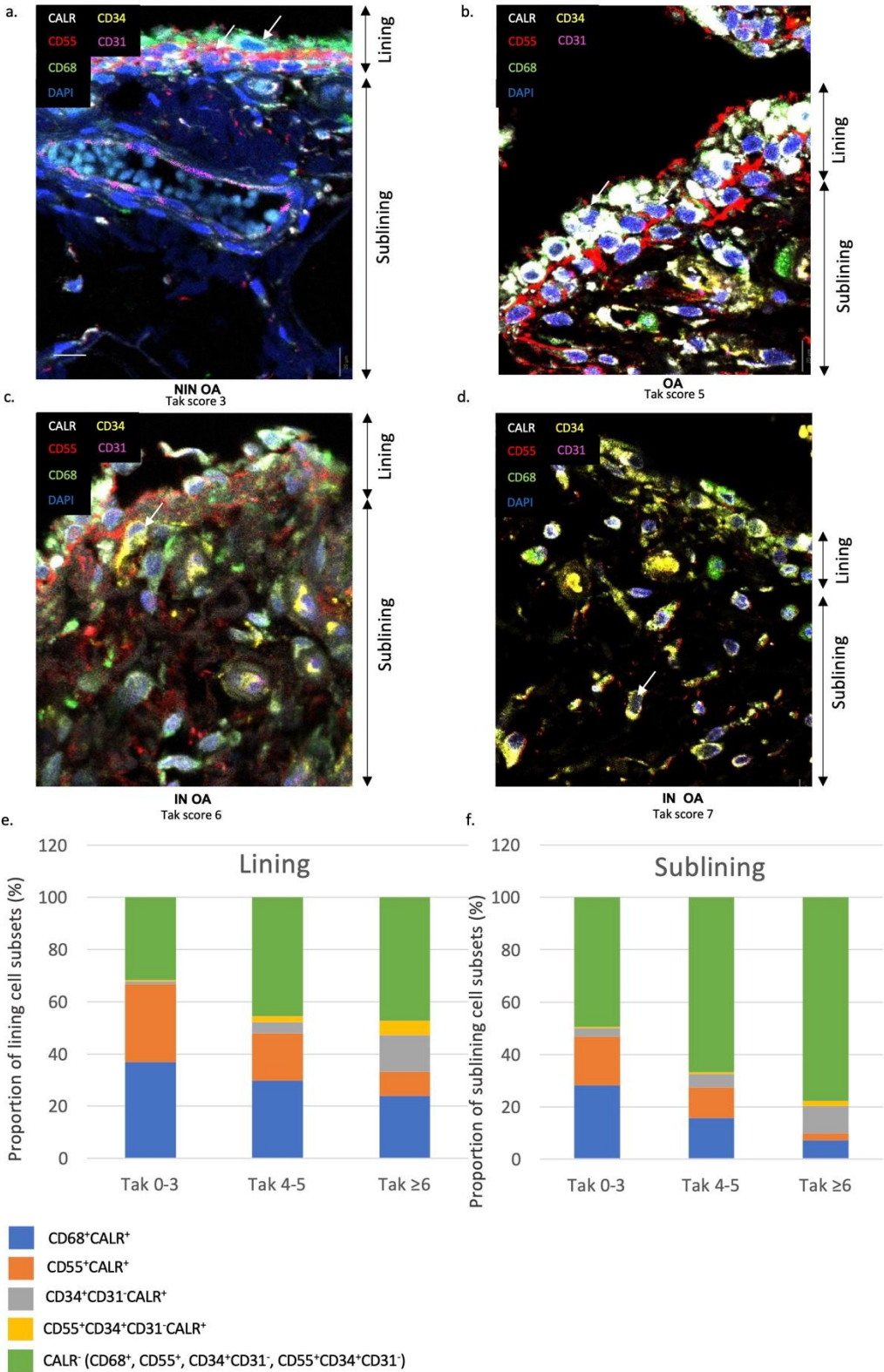
Supplementary Figure 2. Localization of 7 out of the 11 ER stress proteins in synovial membranes provided from healthy and collagenase-induced synovitis (CIS) mouse model. a) Immunohistochemistry illustrations of 7 of the 11 ER stress proteins of interest provided from FFPE synovial membranes of healthy mouse knees (n=11) and their corresponding collagenase-induced synovitis (CIS) mouse knees (n=11); and b) their quantification (optical density values) obtained by QuPath. Data were analyzed with Prism 9 software (GraphPad). Statistical differences were considered significant for p-values < 0.05 (*p<0.05, **p<0.01, ***p<0.001). The nonparametric paired Wilcoxon test was applied on our data. Highly positive cells have an OD > 0.6 (red spot), moderately positive 0.4 > OD > 0.6 (orange spot), weakly positive 0.2 > OD > 0.4 (yellow spot) and negative cells < 0.2 (blue spot). Graphs illustrate the median of the data. c) Correlation coefficients between the 7 ER stress protein levels measured by IHC with *Il6*, *Il1b*, *Acta2* mRNA levels and synovial thickness, according to the nonparametric Spearman test. Each point represents one biopsy (one mouse), with paired healthy and CIS samples from the same animal. ER: endoplasmic reticulum; FFPE: formalin-fixed paraffin-embedded, OD: optical density; IHC: immunohistochemistry

Supplementary Figure 3



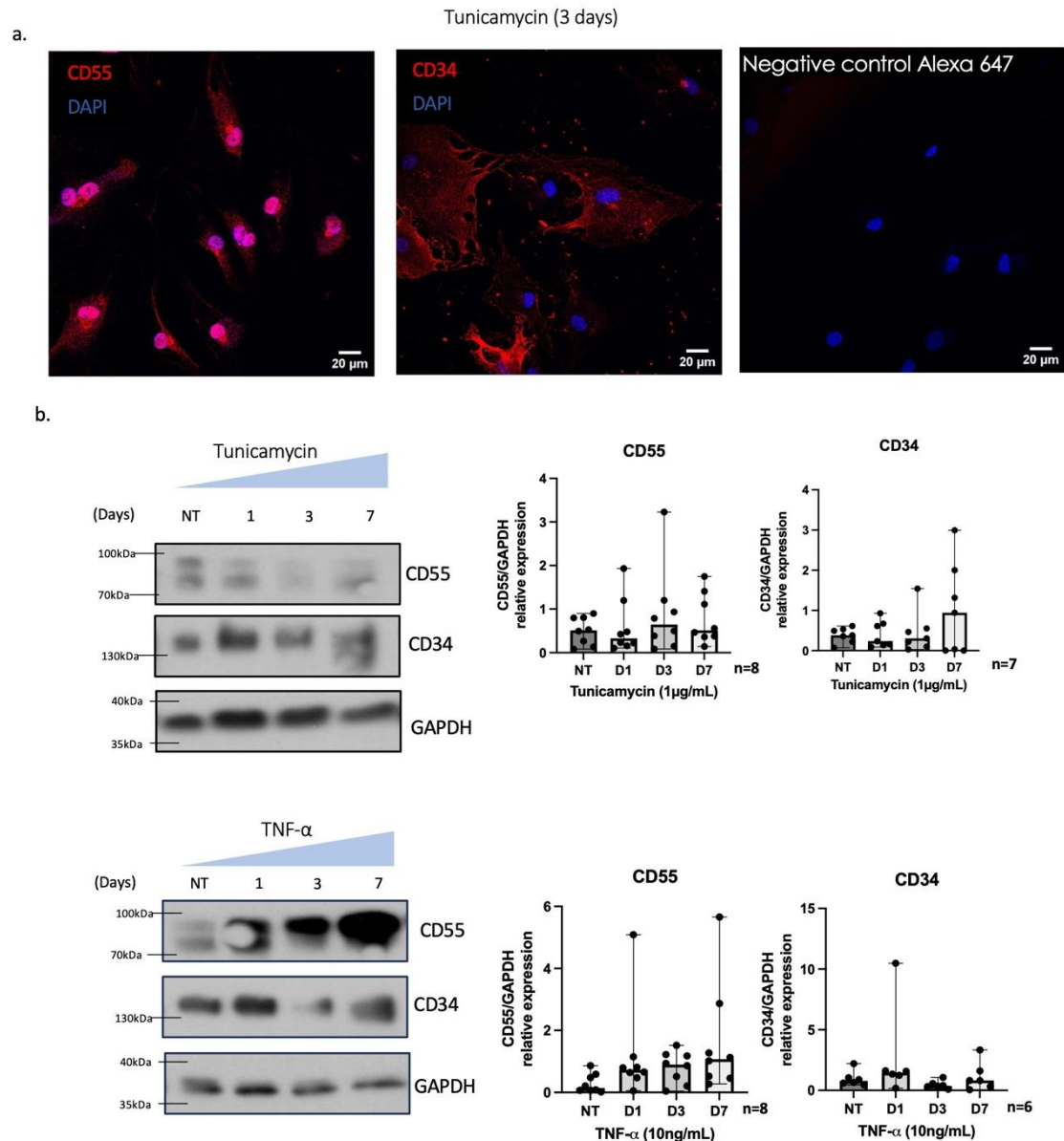
Supplementary Figure 3. Co-expression of PDIA4, GANAB, MANF, CALR and co-expression of PDIA4 FLS and/or MLS in NIN, IN OA and RA. Multiparametric imaging mass cytometry (Cohort 2) representing i) left panel – co-expression of PDIA4 (white), CALR (red), MANF (blue) and GANAB (green); ii) right panel, the co-expression of FLS (CD55 red , CD34 yellow), MLS (CD68 blue) markers with PDIA4 in NIN, IN OA and RA. FLS: fibroblast-like synoviocytes; MLS: macrophage-like synoviocytes; NIN: non-inflammatory; IN: inflammatory; OA: osteoarthritis; RA: rheumatoid arthritis

Supplementary Figure 4



Supplementary Figure 4. CALR expression in synovial compartments. a-d) Representative IF images across increasing Tak scores. Co-staining: CALR, CD55, CD34, CD31, CD68; nuclei (DAPI). Lining and sublining indicated. e-f) Quantification of CALR⁻ cell subsets within the e) lining and f) sublining compartments across Tak score groups. IF: immunofluorescence

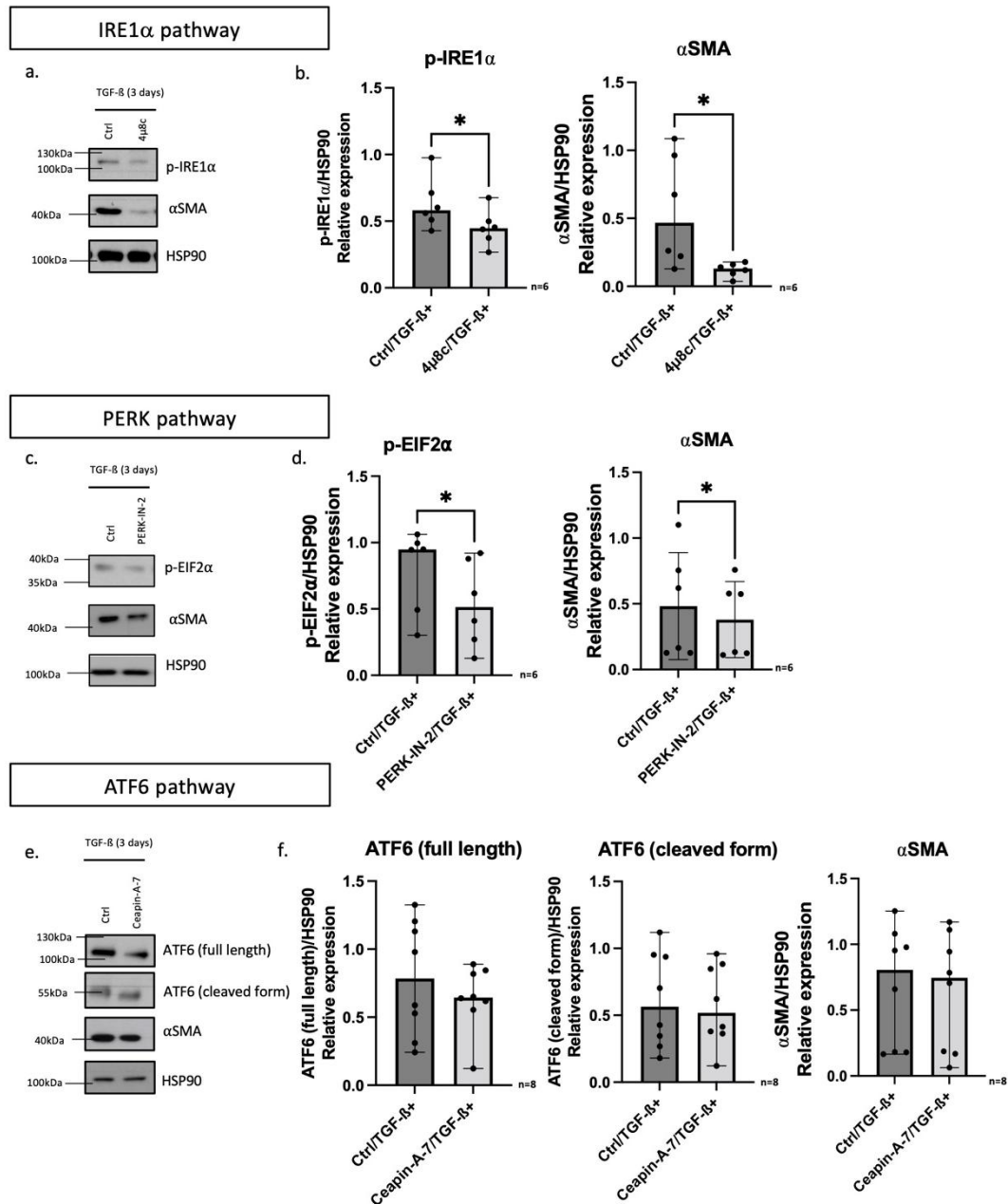
Supplementary Figure 5



Supplementary Figure 5. Characterization of CD55 and CD34 expression in osteoarthritic synovial fibroblasts (FLS).

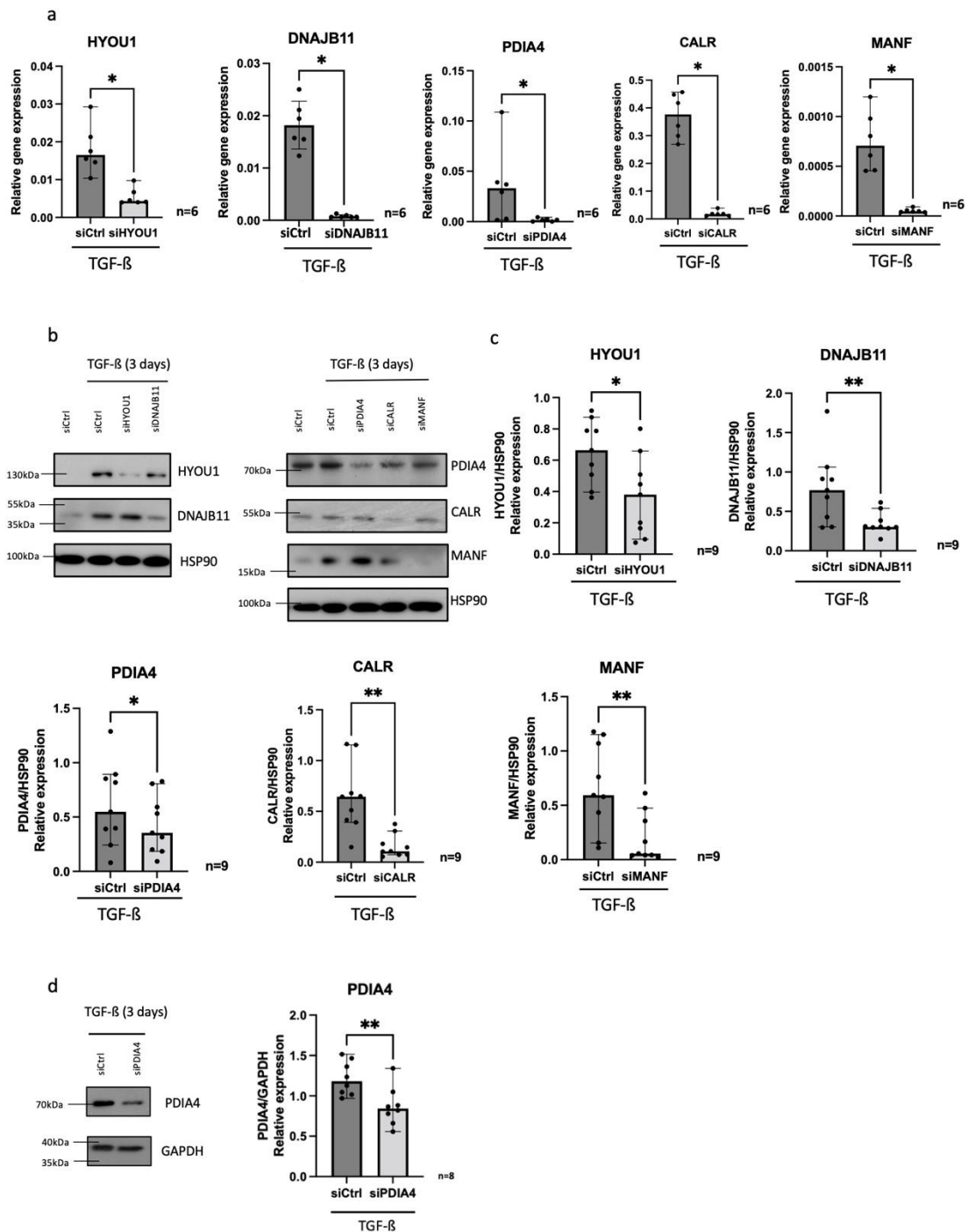
a) Representative immunofluorescence images confirm the expression of CD55 and CD34 in OA-derived FLS following stimulation with tunicamycin (1 μg/mL) for 3 days. Nuclei were counterstained with DAPI. Negative control indicates the absence of nonspecific signal. b) Representative western blot images, their corresponding semi-quantification obtained on FLS after increasing timing of stimulation (1, 3 or 7 days) with Tunicamycin (1 μg/mL) or TNF-α (10 ng/mL) to induce an ER stress or pro-inflammatory process, respectively. Statistical analysis was performed using the nonparametric paired Friedman test followed by Dunn's post hoc test for multiple comparisons. Graphs illustrate the median with a 95% confidence interval of the data. Each point represents one patient under a given stimulation condition. n indicates the number of independent patients. FLS: fibroblast-like synoviocytes; TNF-α: tumor-necrosis factor α;

Supplementary Figure 6



Supplementary Figure 6. UPR pathway modulation and α SMA expression in OA FLS. Representative Western blots showing a) p-IRE1 α , c) p-eIF2 α (PERK pathway), and e) ATF6 (full-length and cleaved forms) in OA FLS treated with 3-days TGF- β and subjected to pathway-specific inhibition using 4 μ 8C (IRE1 α inhibitor), PERK-IN-2 (PERK inhibitor), or Ceapin-A7 (ATF6 inhibitor). α SMA and HSP90 are shown as indicated. Quantification of b) p-IRE1 α d) p-eIF2 α and f) ATF6 (full-length and cleaved), and α SMA expression normalized to HSP90. Each point represents one measurement obtained from an individual patient under a specific stimulation condition; n indicates the number of independent patients. Statistical analysis was performed using a paired nonparametric Wilcoxon test. Statistical differences were considered significant for p-values < 0.05 (*p<0.05). Graphs illustrate the median with a 95% confidence interval of the data. UPR: unfolded protein response; OA: osteoarthritis; FLS: fibroblast-like synoviocytes

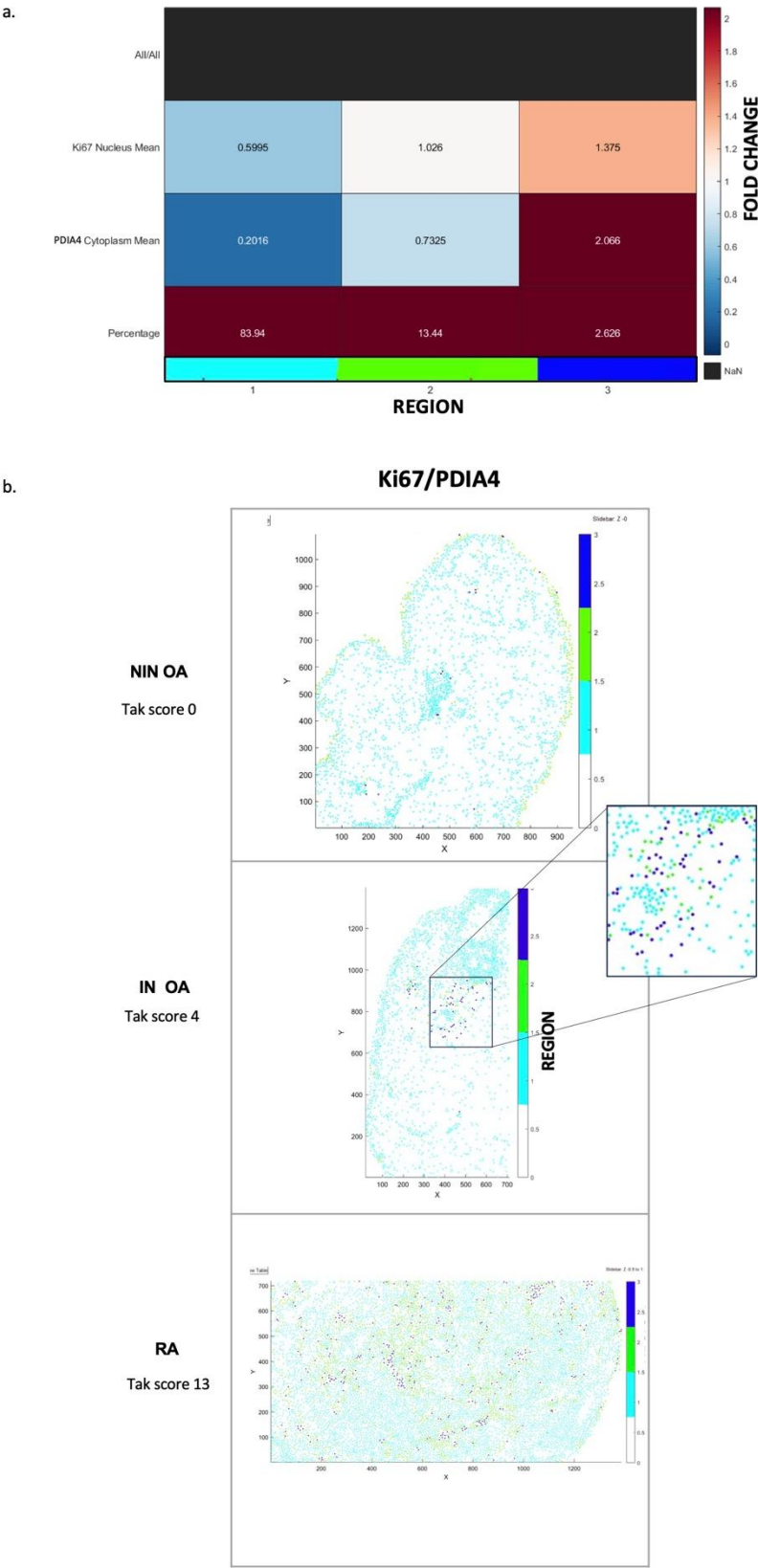
Supplementary - Figure 7



Supplementary Figure 7. ER stress protein silencing in OA FLS

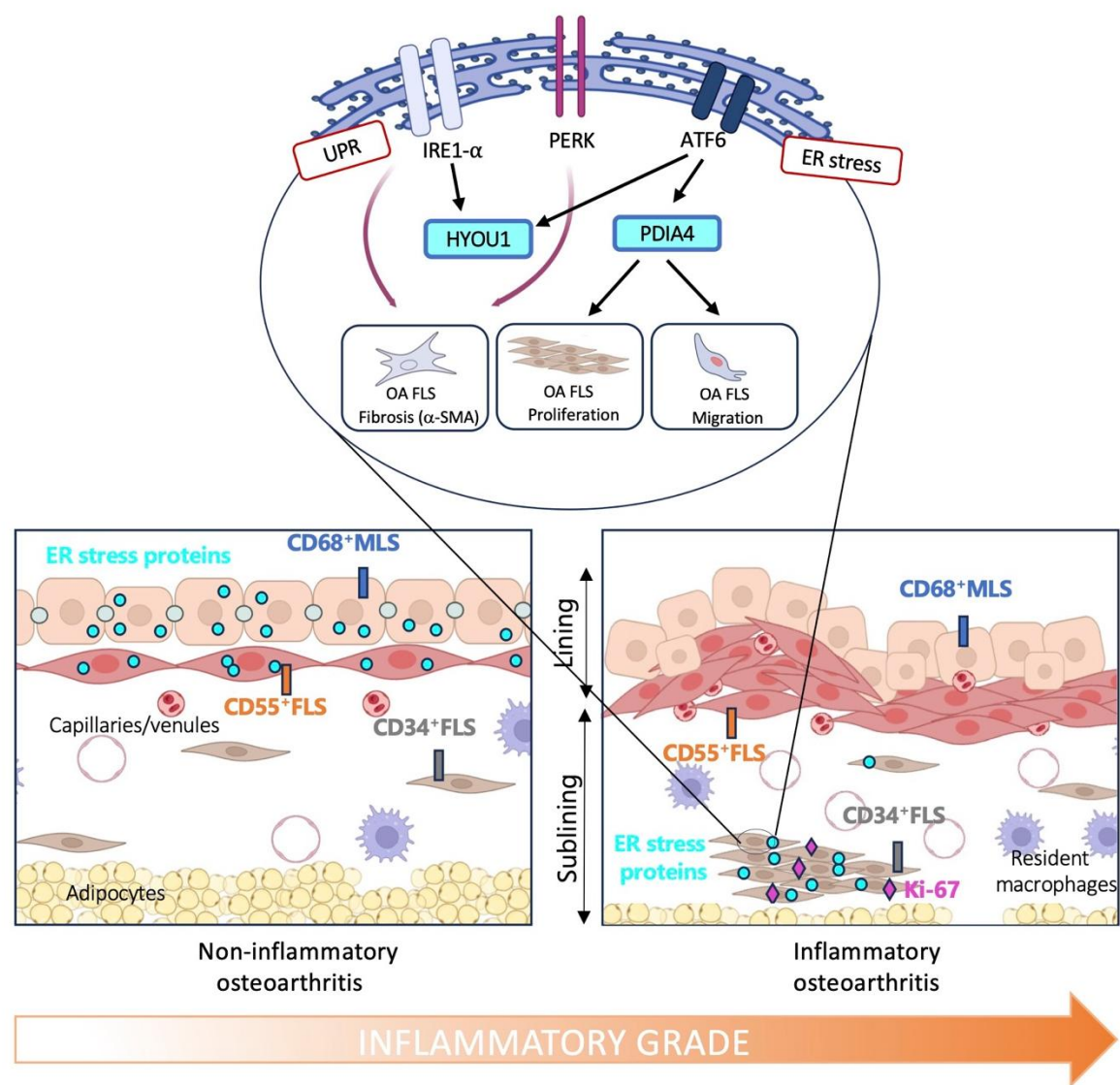
FLS were transfected with siRNAs targeting HYOU1, MANF, PDIA4, DNAJB11, or CALR, then stimulated with TGF- β (10 ng/mL) for 3 days. a) RT-qPCR validation of gene silencing, b) Western blot images showing the depletion of HYOU1, PDIA4, DNAJB11, CALR, and MANF, performed in parallel with the BrdU and MTS assays (c), along with their semi-quantification. d) Western blot images showing the depletion of PDIA4 performed in parallel with the migration assay. HSP90 and GAPDH were used as a loading control. Data are presented as median with 95% CI; each point represents one patient. Statistical significance was assessed using the non-parametric paired Wilcoxon test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Each point represents one measurement obtained from an individual patient under a specific stimulation condition. n indicates the number of independent patients. ER: endoplasmic reticulum; OA: osteoarthritis; FLS: fibroblast-like synoviocytes; Ctrl: controls; TGF- β : transforming-growth factor- β ; siRNA: small interfering; BrdU: bromodésoxyuridine.

Supplementary Figure 8



Supplementary Figure 8. a) Supervised clustering analysis of PDIA4 expression within Ki67⁺ cell populations. b) Spatial representation of supervised clustering analysis of PDIA4 co-expression with Ki67 in non-inflammatory or inflammatory OA, and RA. Blue dots indicate areas with high co-expression of PDIA4 with Ki67, green dots indicate intermediate co-expression, and cyan areas correspond to regions with no co-expression, as defined by the clustering algorithm.

Graphical abstract



Thesis submitted to obtain the degree of
Doctor in Medical Sciences

Academic year 2025-2026