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Department of Pathophysiology, Institute of Biomedicine and Translational Medicine, Faculty of Medicine, University of Tartu, Estonia

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Supervisors: Professor Aare Martson, MD, PhD
Department of Traumatology and Orthopedics,
Institute of Clinical Medicine, University of Tartu, Estonia

Professor Sulev Koks, MD, PhD, FFScRCPA
Perron Institute for Neurological and Translational Sciences,
Nedlands, WA Australia

Professor Abhay Harsulkar, PhD
Department of Pharmaceutical Biotechnology,
Poona College of Pharmacy,
Bharati Vidyapeeth University, Pune, India

Reviewers: Professor, Margus Lember, MD
Dean, Institute of Clinical Medicine,
Department of Internal Medicine, Head of Department,
Professor of Propaedeutics of Internal Medicine,
University of Tartu, Estonia

Dr. Koit Reimand, PhD
Department of Immunology,
Institute of Biomedicine and Translational Medicine,
University of Tartu, Estonia

Opponent: Professor, Kalervo Väänänen, MD, PhD
Institute of Biomedicine,
University of Turku, Turku, Finland

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LIST OF ABBREVIATION

A
ABC – ammonium bicarbonate
ACE – angiotensin-converting enzyme
ACTB – β-actin
ACTBL2 – actin beta like 2
ACTN1 – actinin alpha 1
ACTN4 – actinin alpha 4
ACTR2 – actin related protein 2
ACTR3 – actin related protein 3
ADAMTS – a disintegrin and metalloproteinase with thrombospondin motifs
AGE – advanced glycation end products
APOB – Apolipoprotein B
ARPC3 – actin related protein 2/3 complex subunit 3
AZU1 – azurocidin 1

B
bFGF/FGF-2 – basic fibroblast growth factor
BMP – bone morphogenetic protein
BNIP3 – BCL2 Interacting Protein 3

C
C4a – complement C4A
CADM2 – cell adhesion molecule 2
CCL-18 – C-C Motif Chemokine Ligand 18
CI – confidence interval
CMA1 – chymase 1
COL1A1 – collagen type-I alpha-1 chain
COX-2 – cyclo-oxygenase-2
CP – ceruloplasmin
CPA3 – carboxypeptidase
CPQ – carboxypeptidase Q
CRP – C-reactive protein
CTSB – cathepsin B
CTSD – cathepsin D
CTSG – cathepsin G
CTSL1 – cathepsin L

D
DAMPs – damage associated molecular patterns
DC – deoxycholate
DKK2 – Dickkopf WNT Signaling Pathway Inhibitor 2
D2M – type 2 diabetes

DPBS – dulbecco's phosphate buffered saline

E
ECM – extracellular matrix
EDTA – ethylenediamine tetra-acetic acid

F
FBS – fetal bovine serum
FcERI – high-affinity IgE receptor
FcGR – Fc-gamma receptor
FDR – false discovery rate
FLNA – filamin A

G
GBD – global burden of disease

H
H1-10 – H1.10 linker histone
H1-2 – H1.2 linker histone
H1-5 – H1.5 linker histone
H2A – histone H2A
H2A2B – histone H2A type 2-B
H2A2C – histone H2A type 2-C
H2AFY – H2A histone family
H2B – histone H2B-like
H3F3A1 – uncharacterized protein
H3F3A2 – uncharacterized protein
H3F3 – histone H3.3
HGF – hepatocyte growth factor
HLA-DR – human leukocyte antigen-DR isotype
HSCs – hematopoietic stem cells
HSPA8 – heat shock protein family A (Hsp70) member 8

I
IGFBP2 – insulin like growth factor binding protein 2
IGFBP7 – insulin like growth factor binding protein 7
IGKV1-13 – immunoglobulin kappa variable 1-13
IGKV2-40 – immunoglobulin kappa variable 2-40
IGKV6-21 – immunoglobulin kappa variable 6-21
IGLV1-44 – immunoglobulin lambda variable 1-44
IGLV2-11 – immunoglobulin lambda variable 2-11
IGLV2-18 – immunoglobulin lambda variable 2-18
IGLV3-1 – immunoglobulin lambda variable 3-1
IGLV4-69 – immunoglobulin lambda variable 4-69
IHC – immunohistochemistry

IL-1β – interleukin 1β
IFN-γ – interferon-γ
IL-1RA – IL-1 receptor antagonist
IL1RL1 – interleukin 1 receptor-like 1
ISLR – immunoglobulin superfamily containing leucine rich repeat

J
JNK – c-Jun N-terminal kinase

K
KL – Kellgren-Lawrence

L
LDL – low density lipoprotein
LIF – leukemia inhibitory factor
LogFC – log fold-change
LOX-1 – lectin-like ox-LDL receptor-1
LTBP2 – latent transforming growth factor beta binding protein 2
LTBP4 – latent transforming growth factor beta binding protein 4

M
MAPK – mitogen-activated protein kinase
MAP2K1 – mitogen-activated protein kinase kinase 1
MCPs – mast cell precursors
MCP-1 – monocyte chemoattractant protein-1
METAP2 – methionyl aminopeptidase 2
MIF – macrophage migration inhibitory factor
MMP – matrix metalloproteinase
MRGPRX2 – MAS related G protein-coupled receptor-X2
MS4A2 – membrane-spanning 4-domains A2
MT-MMP – Membrane Type1-MMP

N
NCCS – National Centre for Cell Science
NEDD – N, N-naphthylethylenediaminedihydrochloride
NGF – nerve growth factor
NN – nitrate-nitrite
NO – nitric oxide

O
OA – osteoarthritis / osteoartriit (Estonian translation)
OGFR – opioid growth factor receptor

P
PAFAH1B2 – platelet activating factor acetylhydrolase 1b catalytic subunit 2
PBS – phosphate buffered saline
PGE2 – prostaglandin E2
PIAS4 – Protein Inhibitor of Activated STAT 4
PIIANP – type IIA procollagen N-terminal propeptide
PMA – phorbol 12-myristate 13-acetate
PLA2 – phospholipase A2
PLBD1 – phospholipase B domain containing 1PL2G2A – phospholipase A2 group IIA
PLS-DA – Partial least square discriminant analysis

Q
qRT-PCR – quantitative reverse polymerase chain reaction

R
RA – rheumatoid arthritis
RAB7A – RAB7A, member RAS oncogene family
RAGE – receptor of advanced glycation end products
RANKL/NFκB – receptor activator of nuclear factor-kappa-B ligand
RIN – RNA integrity number
RNTEP – arginyl aminopeptidase
ROS – reactive oxygen species
RPMI – Roswell Park Memorial Institute (cell culture medium)
RT – room temprature

S
SA – sulphanilic acid
SASP – senescent-associated secretory phenotype
SELE – E selectin
SF – synovial fluid
SM – sünoviaalmembraan (Estonian translation)
SV – sünoviaalvedelik (Estonian translation)
STAT4 – Signal Transducer and Activator of Transcription 4

T
TCA – trichoroacetic acid
TFA – trifluoro acetic acid
TGF-β – transforming growth factor-β
TGFBR3 – transforming growth factor beta receptor 3
THP1 – human monocyte cell line
TIMP – tissue inhibitor of metalloproteinase
TLR – toll-like receptor
TMEFF2 – tomoregulin-2
TNF– tumor necrosis factor

TPSAB1 – Tryptase Alpha/Beta1
TRAF2 – TNF Receptor Associated Factor 2

U
UC – untreated control
U937 – human monocyte-like cells

V
VEGF – vascular endothelial growth factor

W
WOMAC – Western Ontario and McMaster University Osteoarthritis Index

X
XPNPEP1 – X-prolyl aminopeptidase 1

Y
YLD – years lived with disability

LIST OF PUBLICATIONS:

1. S. Koppikar, **P. Kulkarni**, D. Ingale, D. Shinde, N Wagh, S. Deshpande, A. Moghe, P. Ranjekar, A. Harsulkar. Inflammatory response of cultured rat synoviocytes challenged with synovial fluid from osteoarthritis patients correlates with their radiographic grading: a pilot study. In. Vitro. Cell. Dev. Biol. Anim. 2015, 51 (8), 843–850.
2. **P. Kulkarni**, A. Martson, R. Vidya. S. Chitnavis, A. Harsulkar. Pathophysiological landscape of osteoarthritis. *Adv. Clin. Chem*. 2021, 100, 37–90.
3. D. Ingale, **P. Kulkarni**, A. Electricwala, A. Moghe, S. Kamyab, S. Jagtap, A. Martson, S. Koks, A. Harsulkar. Synovium-synovial fluid axis in osteoarthritis pathology: A key regulator of the cartilage degradation process. *Genes*. 2021, 12 (7), 989.
4. **P. Kulkarni**, A. Harsulkar, AG. Martson, S. Suutre, A. Martson, S. Koks. Mast cells differentiated in synovial fluid and resident in osteophytes exalt the inflammatory pathology in osteoarthritis. *Int. J. Mol. Sci*. 2022, 23 (1), 541.
5. **P. Kulkarni**, V. Srivastava, K. Tootsi, A. Electricwala, A. Kharat, R. Bhonde, S. Koks, A. Martson and A. Harsulkar. Synovial fluid in knee osteoarthritis extends pro-inflammatory niche for macrophage polarization. *Mediat. Inflamm*. 2022 (submitted).

Contributions of the author to the original articles:

Publication 1: participation in the study designing, performing cell work, data analysis and interpretation, manuscript drafting

Publication 2: conceiving the study, performing *in vitro* studies and related laboratory work, data interpretation and manuscript drafting

Publication 3: conceiving the study and participation in the study designing, performing cell work, data analysis and interpretation, manuscript designing and drafting

Publication 4: conceiving the study, performing cell work, transcriptome and proteome analysis and data interpretation, manuscript drafting and editing

Publication 5: conceiving the study and study designing, performing cell work, data analysis and interpretation and manuscript writing

1. INTRODUCTION

OA is the most prevalent musculoskeletal disorder in elderly population. It is characterized by joint stiffness, swelling, pain and loss of mobility. As per the Global Burden of Disease Study (GBD) 2019, OA is the 15th highest cause of years lived with disability (YLDs) worldwide, accounting for 2.2% of total global YLDs (18.9 million in 2019). Global OA prevalence is over 7% (528 million people), of which, knee OA is the most commonly occurring disease type that accounts for 365 million cases worldwide and 61% of YLDs lost due to the disease. Knee OA is followed by hand OA (142 million cases and 24% of OA YLDs) and, then hip OA (33 million and 5.5% of OA YLDs) (Leifer et al., 2022). The GBD 2017 revealed that YLDs among the musculoskeletal disorders due to back and neck pain, followed by OA, accounts for around 7.1% of global disease burden. The burden is statistically significant as compared to 2007 of 31.4% [95% confidence interval (CI) 30.7 to 32.1]. From socio-economic point of view, OA is a major public health concern in elderly population, affecting over 70% of seniors aged 65 years and is associated with many direct costs related to disease management, hospitalization, and joint replacement surgeries, as well as indirect costs due to work absenteeism and disability. Overall costs incurred by OA represent 1–2% of GDP worldwide. The incidence of the disease is increasing with ageing population and with rising obesity, as the major risk factor associated with OA. Considering a substantial amount of disease burden caused by OA, Osteoarthritis Research Society International (OARSI) published a White Paper, describing it as a serious disease in 2016 (March et al., 2016).

Despite being one of the costliest and disabling form of arthritis and being more prevalent than other arthritic disorders, to date there is no treatment to cure OA or even to effectively delay its progression so as to improve the patient's quality of life and to ease economic burden. The major challenges in developing effective therapies for OA is its heterogenous nature and obscure pathology. OA pathology is in evolving stage; current state of research defines OA as a low-grade inflammatory disease affecting the whole joint. OA appears to be a result of multifaceted interplay of mechanical, cellular, and systemic molecular factors (Hunter, 2011a) that has systemic and immunoregulatory roles (Malemud, 2015), including several complement proteins that are implicated in chronic-low-grade inflammation (Wang et al., 2011). Whereas involvement of low-grade inflammatory component is accepted in OA, synovial membrane, and synovial fluid (SF) are the main sites of this inflammation (Henrotin et al., 2014, Kulkarni et al., 2021). Being well connected with the systemic circulation, synovium acts as a mediator for translating systemic insults into the disease specific pathology. SF is a local counterpart of synovium that mediates signalling among all the joint tissues and holds disease specific insults from the early stage of OA development. Inflamed synovium is presented with hypertrophy and produces pro-inflammatory and catabolic media-

tors such cytokines, reactive oxygen species (ROS), nitric oxide (NO), prostaglandin E2 (PGE2) and neuropeptides altering cellular metabolism and cartilage matrix remodelling and repair (Sutton et al., 2009). Moreover, the inflamed synovium is also responsible for an excessive production of proteolytic enzymes responsible for cartilage breakdown (Sellam and Berenbaum, 2010). Cartilage degradation products further induce synovial inflammation, creating a feedforward loop that exacerbates clinical symptoms and joint degradation in OA patients (Gierman et al., 2012). Pro-inflammatory mediators produced by chondrocytes and synoviocytes also increase oxidative stress, causing a damage to the joint tissues via ROS mediated pathology (Poulet and Beier, 2016).

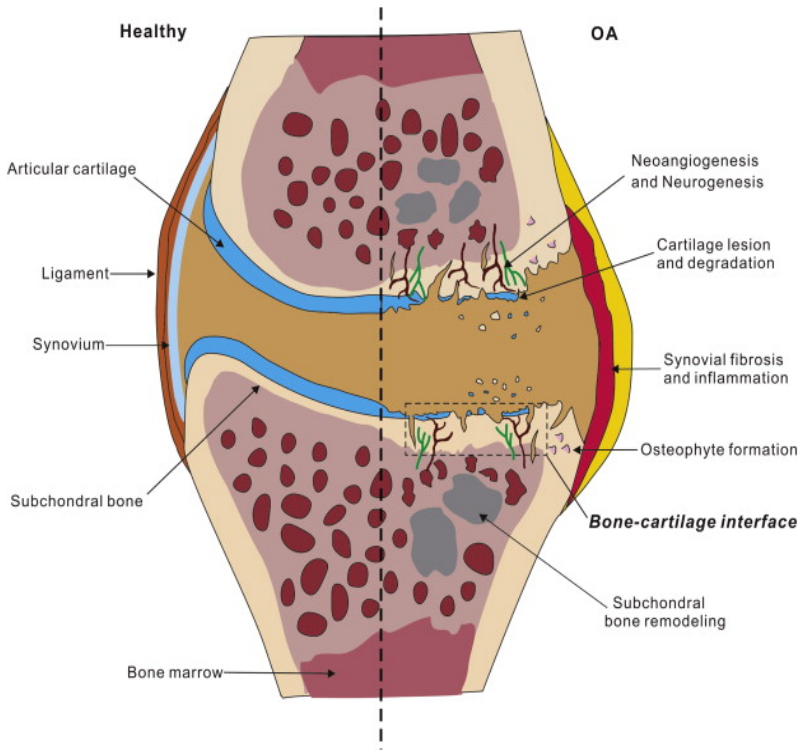
Accounting a key role of inflammation in OA pathology, a systemic blocking of conventional inflammatory pathways (such as anti-TNF or anti-IL1β agents) was thought to be effective in the management; however, it was found ineffective in generalized OA and minimally effective in erosive OA (Verbruggen et al., 2012, Chevalier et al., 2013). This underlines a fact that inflammatory mechanism in OA is not fully understood, and more investigation is required. The present thesis was planned to evaluate inflammatory mechanism revolving around the synovium-SF axis in OA pathology. In this regard, a role of synovium has been much more illustrated with microlevel analysis from gene expression studies to single cell-RNA sequencing of the samples. SF analysis however is limited to understand a molecular make-up of the fluid and there is a wider scope for exploring its functional involvement in OA pathology. Therefore, an outlook of the present thesis was to investigate a 'performance-based involvement' of SF beyond merely a 'communication channel' for nutrition and molecular signalling. We designed 'SF-challenge test' and '*in vitro* cell differentiation assay', wherein OA SF samples of different disease severity were used to induce cells and their modulation response was investigated on inflammatory and immunological parameters. Advantage of both the biological assays was their close physiological resemblance to *in vivo* conditions; the cells were induced with SF samples using their natural pathobiological protein make-up at different OA stages and hence were expected to respond similar to *in vivo* conditions. Simultaneously, proteome analysis and immunophenotyping of SF cells was performed to support the outcomes from the biological assays. On the other side, synovium genes expression studies were undertaken to map the inflammation pattern at various stages of OA. Osteophytes are an integral part of OA joints; however, research on osteophytes is fragmentary, obscure and has not been attempted enough to reveal molecular and cellular events associated with it, especially in the context of OA pathology. The author undertook this task in her PhD tenure in order to bridge this gap and performed RNA-seq analysis of fully grown osteophytes. The RNA-seq analysis, followed by a functional analysis of the gene expression patten that revealed an active involvement of immune cells and a role of SF in immune cells differentiation and maturation, is a new dimension of OA pathology and should prove worthy for taking into consideration for designing appropriate therapies.

2. REVIEW OF LITERATURE

2.1 OA pathology

OA pathology is not fully understood and still unfolding. Conventionally, OA was considered as a 'wear and tear' or 'aging' disease till the end of twentieth century. It was considered as a net result of any process leading to an increased pressure of weight-bearing joints of the body or fragility of cartilage matrix (genetic alterations of matrix components). This understanding was based on the observation that chondrocytes, the only cell type present in articular cartilage, have a very low metabolic activity and no ability to repair the damage. Also, unlike all other tissue types, articular cartilage is anural and avascular and once damaged cannot be repaired (Berenbaum, 2013).

With improved knowledge on OA pathology, now it is put forward as a disease of the 'whole joint', wherein all the joint tissues like synovium, subchondral bone, tendons, and ligaments undergo pathological alterations along with the articular cartilage (Bijlsma et al., 2011, Hunter, 2011b). Out of all, cartilage, subchondral bone, and synovium are believed to be the main players in the initiation and propagation of OA, however the exact role of these three may vary from patient to patient. Imbalance between pro-inflammatory and anti-inflammatory cytokines is a pre-requisite for the development of all forms of OA that result in hallmark features of the disease like cartilage and bone degradation, synovitis and osteophytes formation (Sellam and Berenbaum, 2010). OA development process maybe initiated by releasing cartilage degradation fragments into the joint cavity that leads to synovial inflammation triggered by toll-like receptors (TLRs) (Hunter, 2011b, Little and Hunter, 2013). In some patients on the other hand, the development process begins with synovial inflammation that results in the release of pro-inflammatory and pro-degradative mediators like, cytokines, lipid mediators, neuropeptides, and matrix metalloproteinases (MMPs) (Hunter, 2011b, Little and Hunter, 2013). The pro-degradation mediators like MMPs further degrade the cartilage, contributing to a cycle of inflammation and the disease worsening (Hunter, 2011b, Little and Hunter, 2013). As a consequence of abnormal loading, subchondral bone remodeling can lead to cartilage degradation (Hügle and Geurts, 2017). Subchondral bone is also seen as one of the sources of inflammatory and pro-degradative mediators, which contribute to cartilage degradation and inflammation (Sanchez et al., 2012, Berenbaum, 2013, Houard et al., 2013).



(Yuan et al., 2014)

Figure 1: A schematic diagram comparing a healthy and osteoarthritic joint**.** OA is a whole joint disorder, wherein all the joint tissues undergo pathological alterations. Synovium-SF-cartilage axis is at the center in OA pathogenesis. A complex interplay among oxidative stress, cartilage degeneration and synovitis is responsible for driving OA pathogenesis (Mobasheri et al., 2017) and is summarized below.

2.1.1 ROS and oxidative stress

In a healthy joint, low levels of ROS contribute for maintaining cartilage homeostasis by modulating chondrocyte apoptosis, gene expression, cytokine production and extracellular matrix (ECM) synthesis as well as its breakdown (Henrotin et al., 2003). Superficial and middle zone of healthy cartilage receives oxygen through diffusion, where mitochondria in chondrocytes respire producing ROS (Lee and Urban, 1997). In pathological condition like OA, oxygen tension in SF is subjected to fluctuate due to ischemia, accelerated joint tissue metabolism and sustained strain. Also, chondrocytes produce excess of ROS in response to partial oxygen pressure and mechanical stress. Excess of ROS generate other free radicals (like ONOO and H2O2) and triggers an oxidative damage to cellular proteins, oxidizing lipids, proteins, carbohydrates and DNA in the cartilage (Radi, 1998, Fermor et al., 2007). Over-produced ROS have a

dual action on ECM in the form of accelerated destruction and impaired synthesis. Free radicals of ROS attack directly on proteoglycan and collagen molecules, preventing a formation of collagen fibrils; particularly, OH-degrades collagen and even modifies amino acid composition (Greenwald and Moy, 1979).

2.1.2 Cartilage loss

An irreversible and non-linear loss of the articular cartilage is a characteristic of OA pathology. Excessive MMPs production by the key cytokines like, IL-1β and TNF-α is primarily responsible for cartilage destruction in OA. MMP-1 and MMP-13 are the major players in ECM degradation. Both the MMPs are the members of collagenase sub-family and primarily degrade type-II collagen (Tchetverikov et al., 2005). In addition to type-II collagen, MMP-13 is also able to degrade aggrecan, a proteoglycan. Synoviocytes and chondrocytes are the main sources of MMP-1 and MMP-13 production. Other than MMP-1 and 13, MMP-2, MMP-3 and MMP-9 are associated with a degradation of non-collagen matrix components of the joint. Of note, in spite of a substrate specificity, each member of collagenase sub-family can effectively compensate each other's function and thus has a potential to cleave the triple helix of collagen. MMP-13 and MMP-1 preferentially cleave type-II and type-III collagen, respectively; MMP-8 is effective against type-I collagen (Elliott et al., 2003). The gelatinases (MMP-2 and 9) are involved in a degradation of denatured collagen/gelatine, while degradation of non-collagen matrix components is partially mediated by stromelysins and matrilysins. Among stromelysins, MMP-3 has a specificity for proteoglycan and further, it also contributes to the activation of proMMP-1 (Unemori et al., 1991). Matrilysin production is induced by TNF-α and IL-1β (Ohta et al., 1998) and they have ability to cleave proteoglycans. MMPs' attack on proteoglycan result in aggrecan losing their G1 domain, which is responsible for the interactions with hyaluronic acid and link protein and further dissociation from the matrix (Sandy et al., 1991). Membrane type1-MMP (MT-MMP) contributes for cartilage loss in OA by activating proMMP-2 and pro-MMP-13, which further degrade collagen, gelatine, and proteoglycans (Honda et al., 2001). Beside MMPs, immune cells including activated macrophages, neutrophils, dendritic cells and mast cells release a spectrum of other proteases like, serratiopeptidase, carboxypeptidase, tryptase and aggrecanase, which contribute significantly towards ECM degradation (Chen et al., 2003).

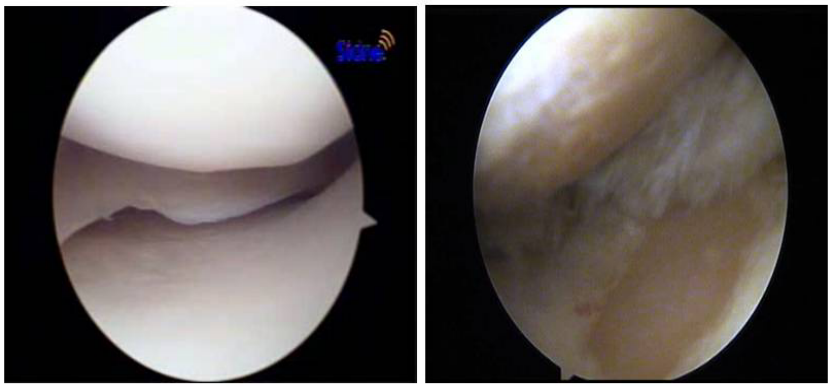


Figure 2: A typical arthroscopic view of human knee joint articular cartilage; A) healthy cartilage B) weathered cartilage in OA.

Although it is known that pro-inflammatory cytokines are primary causative for cartilage loss in OA, the main challenge of OA management is its asymptomatic nature. Since a phase of active cartilage degeneration is elusive to both, physician as well as patient, it is difficult to find an appropriate disease stage for medical intervention. This scenario therefore imposes a need to understand a molecular biochemistry at different OA stages that can consequentially help to determine an appropriate stage of therapeutic intervention and design personalized therapies.

2.1.3 Synovitis, a pivotal pathological event in OA

Over the last few years, a conventional non-inflammatory nature of OA has been challenged (Bonnet and Walsh, 2005, Berenbaum, 2013). Clinical symptoms like joint stiffness, warmth, pain, and joint effusion indicate a presence of inflammation in OA joints (Robinson et al., 2016). Increased levels of cytokines, NO, prostaglandins and MMPs detected in the OA affected joint tissues like synovium, cartilage and SF led to the first step of 'inflammatory' theory. OA synovium is characterized by a massive infiltration of monocytes, production of pro-inflammatory cytokines and the other catabolic mediators, which are responsible for cartilage degeneration (Benito et al., 2005). Systemic oxidative stress induces synoviocytes and chondrocytes to produce IL-6, IL-8, leukemia inhibitory factor (LIF) and prostaglandins (Bondeson et al., 2010). Chronic inflamed synovitis also acts as a stimulator for release of MMP-1, MMP-3, MMP-13 (Nuti et al., 2009). High level of C-reactive protein (hsCRP) is common in OA patients and is a reflection of systemic manifestation of chronic synovitis (Pearle et al., 2007). In advance OA cases, synovium reveals a remarkable hypertrophy, hyper vascularization and enervation amounting to the inflamed painful condition.

Inflammation in OA is atypical and of sub-acute type. The inflammatory marker levels in OA affected tissues were higher than healthy controls but were modest in comparison to rheumatoid arthritis (RA) individuals (Sohn et al., 2012). Furthermore, arthroscopy and histological investigation of OA synovium revealed a sub-acute type of inflammation (Pessler et al., 2008). Immune cells infiltration (like macrophages and T cells) in OA synovium was found much lower as compared to RA synovium (Haseeb and Haqqi, 2013). Importantly, mononuclear immune cells infiltration and overexpression of the inflammatory mediators in synovium are seen in early OA and predate radiographic damage in OA (Sokolove and Lepus, 2013). Predisposition of metabolic disorders like obesity and diabetes to OA also suggests that systemic inflammatory factors may play an important role in the pathology (Courties et al., 2017). These evidences successfully established a strong involvement of chronic low-grade inflammation in OA pathology.

2.1.4 Involvement of immune system in OA inflammation

An interplay between mechanical damage and low-grade chronic inflammation in OA pathology intricately involved an activation of innate as well as adaptive immune responses in the initiation and sustaining the inflammation (Orlowsky and Kraus, 2015). Etiological factors causing initial tissue injury, release several cartilage-specific auto-antigens, which trigger activation of immune responses. Also, immune cells including macrophages, T cells and B cells that infiltrate OA synovium are the main contributor for a release of disease specific cytokines and chemokines. These cytokines are responsible for an excessive production of cartilage degrading factors such as MMPs and PGE2. Presence of immunoglobulin and immune complexes against cartilage components in OA samples like synovium, cartilage and plasma (Jasin, 1988) and activation of complement system (Wang et al, 2011) are a piece of evidences for activated immune system in OA pathology.

Macrophages are the most prominent immune cells in inflamed synovium (Bondeson et al., 2006). Although their number is lower in OA as compared to RA synovium, the cells are highly activated and contribute significantly in OA progression by releasing a plethora of cytokines, which play a pivotal role in the pathology (Hogg et al, 1985). From a clinical perspective, several studies reported a positive correlation between macrophages and OA associated pain, osteophytes and the disease severity (Blom et al., 2004, Blom et al., 2014, Daghestani et al., 2015). Macrophages respond to stimuli by modifying their phenotype and function. Because of this unique characteristic, macrophages are a crucial player in initiation and resolution of inflammation (Van Tiel et al., 2016). These cells are broadly categorized into two types – ‘M1 type’, which are classically activated and ‘M2 type’, which are alternatively activated (Wang et al. 2014). Activated M1 macrophages further stimulate Th1 cells and further release pro-inflammatory cytokines including, IL-12, IL-23, TNF-α, IL-1 and NO. On the other hand, activated M2 macrophages stimulate Th2 cell and are

involved in the release of IL-10, CCL18, TGF-β and IL-1 receptor antagonist (IL-1RA) (Wang et al. 2014, Van Tiel et al., 2016). Sequentially, the cytokines produced by Th1 and Th2 cells act as a stimulator for M1 and M2 cells, respectively (Xue et al.,2014, Van Tiel et al., 2016,). Conventionally, M1 and M2 types are considered pro-inflammatory and anti-inflammatory, respectively. Research studies, however, have reported a positive association between M2 macrophages and chronic inflammation (Martinez and Gordon, 2014, Xue et al.,2014,). These cells were showed to produce pro-inflammatory cytokines in a synovial microenvironment with autoantibodies in RA patients (Vogelpoel et al., 2014). In OA, the mediators that cause cartilage damage, like DAMPs and ECM components exude into SF and activate synovial macrophages (Sohn et al., 2012). Activated synovial macrophages eventually stimulate other immune cells, like CD4+ T cells and pro-inflammatory cytokines production (IL-1β and TNF-α) (Saito et al., 2002, Blom et al., 2004, Blom et al., 2007). Research studies reported that, a combination of M1 and M2 macrophages are present in OA synovium (Oehler et al., 2002, Blom et al., 2004, Manferdini et al., 2016); however, Utomo et al., 2016 showed that majority of inflamed synovial macrophages are of M2 type. A location of macrophages also influences the phenotype expressions in OA. For example, CD80+ M1 and CD206+ M2 macrophages were seen predominantly in synovium lining and sub-lining layer, respectively (Fahy et al, 2014).

Besides induction and maintenance of the synovial inflammation, macrophages are actively involved in cartilage degradation, osteophytes formation and joint pain induction in OA pathology. Pro-inflammatory cytokines and mediators produced by macrophages, are directly responsible for a disrupted chondrocytes metabolism, down-regulation of associated anabolic processes in the joint environment and excessive production of MMPs and aggrecanase. Depletion of synovial macrophages caused a significant reduction in IL-1β and TNF-α levels and sequentially, cartilage loss (Bondeson et al., 2006). The depletion of synovial macrophages also revealed a reduction in MMP-2, MMP-3, and MMP-9 (Goldring, 2000, Bondeson et al., 2006, Bondeson et al., 2010) and thus, confirmed their active participation in excessive MMPs production. Also, macrophages induced ROS, hypochlorous acid, chlorine gas and chlorinated peptides were observed exerting oxidative damage on articular cartilage (Steinbeck et al., 2007). The *in vitro* explant model, which tested an impact of macrophages factors on cartilage degradation, concluded that pro-inflammatory factors of M1 macrophages induce cartilage disruption. Further, the same study reported that, anti-inflammatory M2 factors were ineffective for any direct inhibition of cartilage degeneration or inflammation (Utomo et al. 2016).

Osteophytes are a classic feature of OA joints and are formed as a result of synovial inflammation, caused by macrophages in particular, which are the harbingers of synovitis. Depletion of macrophages cause a significant reduction in osteophytes despite of triple intra-articulate injections of transforming growth factor-β (TGF-β) in a murine model indicated that macrophages play a crucial role in osteophytosis (a process of osteophytes formation). The same study also

demonstrated an essential role of macrophages in the release of TGF-β, bone morphogenic protein-2 (BMP-2) and BMP-4, key players involved in the osteophyte formation (Blom et al., 2004). Inhibition of TGF-β *in vivo* or over-expressing TGF-β antagonist prevented osteophyte formation (Scharstuhl et al., 2002, Scharstuhl et al., 2003). Collagenase induced murine model of OA wherein, osteophytes formation was observed at days 7 and 14 after induction, depletion of macrophages revealed a dramatic reduction in this process up to 84% on 7th day and 66% on 14th day (Bloom et al., 2004). Papen injection induced mouse model of OA showed that triamcinolone acetonide injection provides a complete prevention against osteophyte formation (Daghestani et al., 2015). Of note, triamcinolone acetonide injection induce activation of FRβ/ CD163+ macrophages. Clearly, an induction of M2 (anti-inflammatory) macrophages caused osteophytosis inhibition in this model (Siebelt et al., 2015). Hepatocyte growth factor (HGF) is produced by chondrocytes and known to instigate macrophages to produce TGF-β through monocyte chemoattractant protein (MCP-1) expression and thus, promote osteophyte formation. A study of 41 OA patients reported a positive correlation between HGF and osteophytes formation (Dankbar et al., 2007). Furthermore, sCD163 and sCD14 are macrophage markers and revealed a positive correlation with osteophytes progression (Daghestani et al., 2015).

Inflammation in OA is also referred as a sterile inflammation, an inflammation in the absence of any pathogen. In this context, inflammation in OA closely relates to "inflammaging", (a term coined by fusion form of two words, inflammation and aging) (Franceschi et al., 2000). It is well known that 'ageing' has been a long-standing confounding factor for OA. Chronic and low-grade inflammation associated with the natural aging process could promote OA development (Loser et al., 2016). An association between inflammaging and OA is through a plethora of cytokines. In CHIANTI study, pro-inflammatory as well as anti-inflammatory biomarkers were analyzed simultaneously to understand or predict chronic disease and inflammaging (Morrisette-Thomas et al., 2014). This study reported two important observations – first, pro-inflammatory markers revealed a strong correlation with age and chronic disease status. A subgroup of pro-inflammatory markers was linked to innate immune system activation and did not correlate with age but showed a strong association with chronic diseases and even revealed a protective role, suggesting a more complex interplay among the cytokines (Morrisette-Thomas et al., 2014).

Inflammaging causes a deposition of age-related visceral fat, at least in parts. The increased fat mass along with a declined muscle mass is responsible for elevated adipocytes and macrophages, which release a plethora of cytokines and adipokines that are thought to contribute to OA. Further, this increased fat mass also contributes for metabolic alterations that ultimately result in the increased levels of circulating free fatty acids, hyperglycaemia and oxidative stress. This catabolic environment along with the other confounding factors like obesity, contributes for creating a joint instability, promote matrix destruction and OA development (Courties et al., 2015). Knee infra-patellar fat pad is also a

recognized source of leptin, adipokines, adiponectin, basic fibroblast growth factor (bFGF; also known as FGF-2), vascular endothelial growth factor (VEGF) and inflammatory cytokines like, TNF-α and IL-6; all these biomarkers have an established role in OA pathology (Ushiyama et al., 2003). Ageing is also associated with a marked increase in cell senescence. The dying cells, cell debris and degradation intermediates invariably instigate the activation of macrophages that have a primary responsibility for clearing of these debris and particulate matter.

Augmented infiltration of macrophages in synovium showed an association with joint pain in OA (Bonnet and Walsh, 2005, Kraus et al., 2016). Various links demonstrate this association, for example, in knee OA, serum TNF-α was found correlated with the pain. Synovial macrophages are a chief inducer of TNF-α (Stannus et al., 2013). IL-6 was found associated with the pain in terminal OA cases, although cellular source of IL-6 secretion (whether macrophages or T cells) remained unclear (Imamura et al., 2015). OA patients with KL grades-II, III and IV (moderate to advance OA) reported a positive correlation between pain and macrophage migration inhibitory factor (MIF), a pro-inflammatory cytokine mainly produced by macrophages (Zhang et al., 2016). Nerve growth factor (NGF), which is induced by synovial macrophages is closely linked with OA pain (Takano et al., 2017).

The review of literature clearly highlights an importance of macrophage polarization in all aspects OA pathology including synovitis, cartilage degradation, knee joint pain as well as osteophytes formation. Inhibition of M1 type polarization as well as promoting M2 type polarization could be a key for effective alleviation of OA symptoms. To promote this idea, a better understanding of macrophage polarization agents in OA joints is a welcome step.

2.1.5 Role of SF in OA pathology

SF is a clear, straw-colored and viscous liquid that occur in the synovial joint, which has biomechanical, metabolic and regulatory functions. In a healthy joint, SF acts as a medium for the transport of essential nutrients, enzymes, cytokines and growth factors responsible for cartilage remodeling (Kokebie et al., 2011). OA SF has been particularly of interest to find out vital clues on OA pathology due to its role as a biochemical channel of the joint. Among the various research studies performed on OA SFs, Sohn et al., 2012 identified 108 proteins in OA SF including, plasma proteins, serine protease inhibitors, proteins indicative of cartilage turnover and the proteins involved in inflammation and immunity. This study further reported that the identified plasma proteins contribute for a low-grade inflammation in OA. Kokebie and his team reported elevated levels of IL-6 and IL-11 in OA SF samples; however, the level of inflammation was lower than RA SFs (Kokebie et al., 2011). A study of 47 OA patients revealed a positive correlation between the cytokines like, TNF-α and IL-6 (assessed in knee SFs) and pain and joint function [sub-index of Western Ontario and McMaster University Osteoarthritis Index (WOMAC) scale], respectively (Orita

et al., 2011). In recent times, proteome analysis of 24 OA patients revealed 31 differentially expressed proteins, wherein majority of the proteins were associated with leucocyte migration (Yang et al., 2021) that warrants a deeper investigation. Partial least square discriminant analysis (PLS-DA) of SF from end-stage OA patients produced an eight biomarkers model to classify the end-stage OA with specificity (98.4%), and reliability (97.4%). The biomarker panel included markers like type IIA procollagen N-terminal propeptide (PIIANP), tissue inhibitor of metalloproteinase (TIMP)-1, a disintegrin and metallo-proteinase with thrombospondin motifs 4 (ADAMTS-4), MCP-1, interferon-γ-inducible protein-10 (IP-10) and TGF-β3 (Jayadev et al., 2020). A SWATH-MS analysis on SF samples, obtained from KL grade-IV OA patients (n = 10) reported 93 differentially expressed OA specific proteins, wherein complement C1r and dickkopf WNT signaling pathway inhibitor 2 (DKK2) correlated with OA severity and can be explored as potential biomarkers or drug targets in OA intervention (Liao et al., 2018). SFs were also tested for OA distinguished metabolites profile including phosphatidylcholine, lysophosphatidylcholine, ceramides, myristate derivatives, and carnitine derivatives (Carlson et al., 2018). SF as an inducing agent, was reported to stimulate metabolic activity of SF isolated cells (De Sousa et al., 2019). SF from aseptic prosthesis loosening showed a stimulatory effect on collagen formation and also induced cell proliferation in MG63 osteoblasts (Tsai et al., 2009). Low concentration of bovine SF stimulated a proliferation in rabbit medial cruciate ligament and anterior cruciate ligament and even higher concentrations of SFs did not show any inhibitory effect on the ligament proliferation (Nickerson et al., 1992). Thus, SF proteins, oxidant radicals, tissue degradation products, systemic signals and SF resident cells contributes to the SF inducing potential, influencing a spectrum of joint cells and are likely to modulate their function. Using SFs from the affected patients for *in vitro* testing is therefore an attractive model to study chronic disease pathologies like OA.

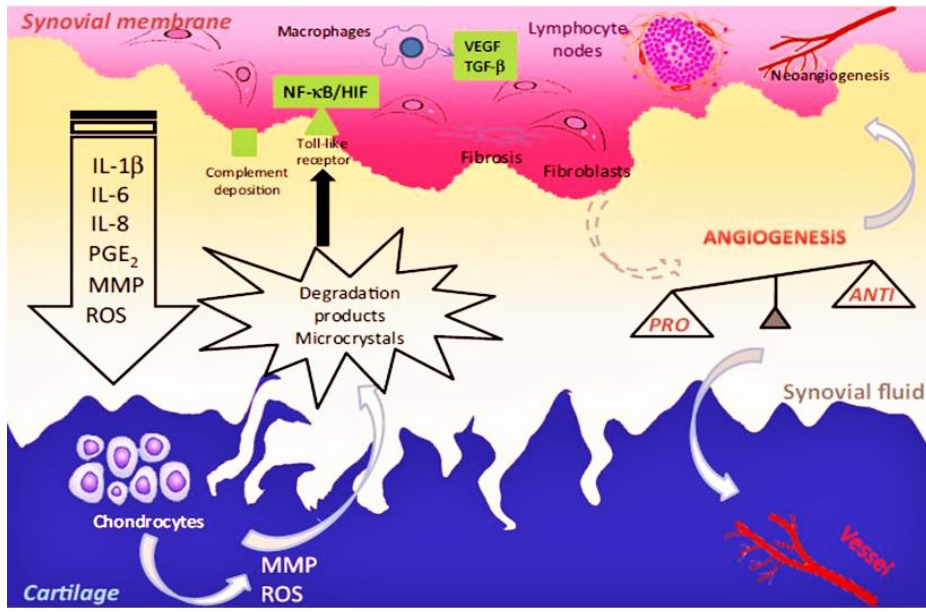


Figure 3: Synovium-SF-cartilage, a central axis in OA pathology. This schematic presentation is an overview of a complex interplay among the key pathological events like, oxidative stress, synovitis, angiogenesis and cartilage degeneration that drives the disease pathogenesis. A vicious circle of ROS, pro-inflammatory cytokines and cartilage degradation products also stimulate TLRs and activation of complement system in OA. (Henrotin et al., 2014)

2.2 OA linkage with the other chronic metabolic disorders

Recent reviews suggest close ties between OA and the other chronic diseases like obesity, type 2 diabetes (D2M), insulin resistance, dyslipidemia and hypertension; chronic low-grade inflammation and insulin resistance are reported as the common links among these co-morbidities (Mobasheri et al., 2017). Thus, metabolic OA is an increasingly recognized OA phenotype (Piva et al., 2015). A common understanding in this regard is, systemic insults of metabolic syndrome induce pathological alterations in skeletal tissues using mesenchymal precursors. Once the skeletal integrity is challenged, cartilage loss overtakes the normal wear and tear process. Thus, cartilage damage remains only a culminating part of entire disease process (Aspden et al., 2001).

Last decade studies present D2M as a risk factor for OA, giving rise to a concept of diabetes-induced OA phenotype (Sellam and Berenbaum, 2013). Insulin resistance induced oxidative stress and pro-inflammatory factors are responsible for an accumulation of advanced glycation end products (AGE), which is associated with alteration of mechanical properties of cartilage, facilitating OA development (Rashid and Haqqi, 2012, Laiguillon et al., 2015,). In OA, chondrocytes are seen to lose their capacity to adapt to local concentration

of glucose, that hampers their differential potential and ultimately cartilage regeneration capacity. Hyperglycemia in diabetes increase synovial angiogenesis by up-regulating VEGF, which further simulates inflammatory pathways in OA pathogenesis (Tsai et al., 2013). From a systemic point of view, leptin, a hormone secreted by adipocytes, is a link between D2M and OA, which plays a role in maintaining a chronic low-grade inflammation (Griffin et al., 2009). Obesity is a well-known confounding factor of OA; excessive weight on the weight-bearing joints results in inhibition of cartilage matrix synthesis and release of pro-inflammatory as well as degradative factors (COX2, IL-1β, TNF-α, MMP-3, 13 and aggrecanase). Adipokines like leptin, which is significantly expressed in OA cartilage and osteophytes, inhibits ECM synthesis (Dumond et al., 2003, Pottie et al., 2006). Infra-patellar fat pad too, is a source of growth factors, cytokines and adipokines, which contribute for the disease development (Ushiyama et al., 2003). Abnormal lipid profile, commonly seen in obesity, contributes for OA development (Klop et al., 2013). While, disruption in HDL metabolic pathway creates an imbalance in cartilage remodeling process along with the other mediators (Triantaphyllidou et al., 2013), high systemic oxidized low-density lipoprotein (ox-LDL) promote joint inflammation and cartilage loss through activation of VEGF and lectin-like ox-LDL receptor-1 (LOX-1) (Kanata et al., 2006). Nevertheless, free fatty acid accumulation in obesity aggravates adipose tissue inflammation by activation of macrophages via TLR-2/4 (Nguyen et al., 2007) and serves as link for systemic involvement in OA. Subchondral bone ischemia and consequentially accelerated apoptosis serve as a link between OA and hypertension. Ischemia of subchondral bone compromises nutrient and gas exchange inducing degenerative changes in the cartilage (Findlay et al., 2007). Apoptosis on the other hand, affects subchondral bone remodeling (Zhuo et al., 2012). Increased bone turnover is reported as a causative factor for cartilage thinning; however, this observation is yet to establish in human OA studies (Berger et al., 2003).

2.3 Summary of the literature review and building of the hypothesis

Once seen as ‘wear and tear’ disease, a growing body of evidence clearly present OA as a disease of whole joint involving not only cartilage but also synovium, subchondral bone, tendons and ligaments. OA pathology is in evolving stage; it is a complex blend of oxidative stress, chronic low-grade inflammation and cartilage degradation engaging cartilage, bone and synovium as key joint tissues and is elaborately discussed above. The exact role of each of these tissues played in the initiation and propagation of OA may differ from patient to patient; this means, in some patients, OA development may be initiated by cartilage degradation leading to release of fragments in the joint cavity. Once these fragments encounter synovial tissue this leads to inflammation. In the other category of patients, especially in post-trauma cases, a

release of pro-inflammatory mediators leads to a production of MMPs, the proteolytic enzymes, by chondrocytes and consequentially degradation of the cartilage, contributing to a cycle of inflammation. Therefore, it is clearly evident that inflammation in OA pathology may be or may not be an initiator, but it becomes a driver of the disease progression at some point.

Many signalling compounds, known as cytokines, are produced by synovium, cartilage, subchondral bone as well as infra-patellar fat pad and are key contributors in the development of all forms of OA. Pro-inflammatory cytokines in excess outweigh the effect of anti-inflammatory cytokines and other anabolic growth factors hampering a delicate balance between catabolic and anabolic processes that fundamentally maintains cartilage homeostasis under healthy conditions. This shift towards catabolism results in cartilage degradation, which together with osteophytes and chronic low-grade synovitis forms the common symptoms and clinical signs of OA. Synovium is a major care provider for articular cartilage and hence, any synovial pathology is bound to cause adverse effect on the cartilage. Synovitis is a scene of inflammation in OA, which receives systemic insults and translate them into the disease specific pathology. Different authors outlined a varying level of significance of synovitis postulating it as responsible for driving the pathogenesis of OA as discussed in detail in this section. Macrophages, T cells and B cells are major infiltrates of OA synovium that release cytokines and chemokines accentuating inflammatory milieu of the joint. Pro-inflammatory cytokines produced by the immune cells are also the stimulators of excessive MMPs production, which is directly responsible for cartilage loss in OA. Non-linear and asymptomatic cartilage loss is one of the major obstacles in developing effective OA therapies. A comprehensive analysis of inflammation pattern at different OA stages (which is directly linked with the cartilage degradation) is a needed approach first to understand the molecular biochemistry in the disease progression and, sequentially to determine the most appropriate disease stage for medical intervention. Also, in the recent years, a key role of macrophage and its polarization towards M1 type has been highlighted in almost every aspect of OA pathology. Therefore, macrophage reprogramming is an emerging target of modulating the inflammation in OA. For effective inhibition of M1 type polarization or promoting M2 type polarization, more illumination is needed on the polarization agents and its mechanism in OA joints.

SF is a synovium's counterpart at local level; it provides nutrition to all joint tissues as well as holds the first insults of OA pathology. A varying level of SF analysis from OA patients exhibited a grade-wise increase in pro-inflammatory factors. Therefore, this fluid is supposed to have a more comprehensive role in OA pathology than it is recognized at present in OA research. In fact, a limited record of studies is also available, wherein a protein make-up of SF obtained from various arthropathies was showed to induce metabolic activity and proliferative changes in various cell types. To outline a functional involvement of SF in OA pathology, we see using SFs from OA patients for *in vitro* testing as an excellent system. Additionally, this model can be equally effective to under-

stand cellular and molecular mechanism in the pathogenesis of chronic diseases like OA, wherein suitable animal models are not available in the current state.

Finally, osteophytes are frequently formed as a result of synovial inflammation. Moreover, they are linked with classical OA symptoms like restricted joint movement, discomfort and pain and therefore, come under a pathological perspective, which are worthy of investigation in a molecular and cellular context to find out their association with OA pathology.

3. AIM AND OBJECTIVES

Overall aim of this PhD thesis was a comprehensive evaluation of inflammatory mechanism revolving around the 'synovium-SF axis' in OA pathology. This included a multi-level analysis of different OA affected tissues such as synovium biopsies and SF samples spanning different degrees of the disease severity and osteophytes. To meet the overall aim, the study modules were planned with the specific objectives as described below:

1. To outline and compare a variation pattern of key inflammatory mediators at different stages of natural OA progression (Publication 1 and 3)
2. To establish a functional involvement of SF in OA pathogenesis in terms of its potential to provide inflammatory microenvironment of knee joint cavity (Publication 1, 2 and 3)
3. To assess an ability of SF to induce immune cell differentiation (Publication 4 and 5)
4. Elucidation of molecular and cellular mechanisms of osteophytes and their linkage with OA pathology (Publication 4)

4. METHODS

4.1 Ethical statement

All the methods performed in this PhD study abide by the Declaration of Helsinki. The study protocols used in this research were approved by the Institutional Ethics Committee of University of Tartu and Institutional Ethics Committee of Medical College, Bharati Vidyapeeth University (282/T13, 227/T-14, 76785 and BVDU/MC/56).

4.2 Patient selection

An indoor or outdoor patient at the Department of Traumatology and Orthopedics, University of Tartu and Bharati Hospital, Bharati Vidyapeeth University, with a confirmed knee OA diagnosis (age group – 40 to 75 years) and excluding the medical conditions mentioned under 'patient exclusion criteria' was considered suitable for the enrollment in the present study. OA diagnosis was performed by an experienced rheumatologist/orthopedic surgeon based on clinical signs, symptoms and radiographic features. The disease severity was determined by Kellgren-Lawrence (KL) radiographic score. KL score system is the most commonly used system for OA grading and is based on the following radiological signs:

1. Grade-I is doubtful narrowing of the joint space and possible, indistinguishable osteophyte
2. Grade-II is definite clearly identifiable osteophytes and possible narrowing of the joint space
3. Grade-III is with moderate multiple osteophytes with definite joint space narrowing, and
4. Grade-IV is marked with large osteophytes with marked narrowing of joint space (Kulkarni et al., 2016).

Patient exclusion criteria

Patients with RA, infected arthritis, OA with tumours, OA with a history of knee joints injuries in past 2 years.

All the recruited patients signed an informed written consent form before participating in the study.

4.3 Collection of clinical samples

4.3.1 Synovial biopsies

In total, twenty-six synovial biopsies were collected from different grades of OA patients, who underwent knee arthroscopy or knee replacement surgery (KL grade-I = 6; KL grade-II = 6; KL grade-III = 6; KL grade-IV = 8). The biopsies

were collected into sterile container in normal saline, immediately transferred to laboratory and stored in liquid nitrogen before they were used for inflammatory biomarkers expression analysis.

4.3.2 SF collection

Knee arthrocentesis was performed for the OA patients with KL grade – I, II and III, who had a knee effusion and required to undergo the procedure to relieve the disease symptoms. The procedure of knee arthrocentesis was performed as described (Mundt and Shanahan, 2010). SF fluid collection protocol was approved by the Institutional Ethics committee (227/T-14 and 76785). The process of arthrocentesis was performed in minor operation theatre under strict aseptic conditions to collect SF samples of KL grade- I, II and III. The fluids from KL grade-IV were collected at the time of knee replacement surgery. Of note, SF sample grading was the same as OA grading. In total, thirty-two SF samples were collected (n = 32); eight SF samples of each KL grade were obtained.

Table 1: an overview of OA-affected clinical samples used in the study

Clinical sample type	Sample number
Synovium biopsies	26 (KL grade-I = 6; KL grade-II = 6; KL grade-III = 6; KL grade-IV = 8)
SF	32 (KL grade-I = 8; KL grade-II = 8; KL grade-III = 8; KL grade-IV = 8)
Osteophytes	6 (KL grade-IV = 5; KL grade-III = 1); patient characteristics and their clinical details are mentioned in Appendix as Table 4

4.4 To outline and compare a variation pattern of key inflammatory mediators at different stages of natural OA progression (Publication 1 and 3)

4.4.1 RNA isolation and qRT-PCR analysis

Total RNA was extracted from the collected synovium tissues using TRIZOL reagent (Invitrogen Co., Carlsbad, CA, USA) and later with PureLink RNA Mini Kit (Invitrogen Co., Carlsbad, CA, USA) or SuperScript-III Cell to cDNA kit (Invitrogen Co., Carlsbad CA, USA) following the manufacturer's instructions. Total RNA was quantified by measurement of UV absorbance at 260 nm. First strand cDNA was synthesized from 1 µg of total RNA using SuperScript First-Strand Synthesis System for qRT-PCR (Invitrogen Co., Carlsbad, CA, USA). qRT-PCR analysis was performed with Applied Biosystems StepOne Real Time PCR System using the following TaqMan gene expression assays (Applied Biosystems, Foster City, CA, USA) and TaqMan

Gene Expression Master Mix (Applied Biosystems, Foster City, CA, USA). mRNA expression amount of the selected markers was normalized against the amount of β-actin, using Step One Software version 2.2.2.

Gene Name	TaqMan Gene Assay number
ACTB	Hs01060665_m1
IL-1 β	Hs00174097_m1
IL-15	Hs01003716_m1
PGE2	Hs00168755_m1
NGF	Hs00171458_m1

4.4.2 Estimation of pro-inflammatory mediators in SF samples

Levels of pro-inflammatory mediators including IL-1β, NO and nitrate-nitrite (NN) were estimated in 20 SF samples (5 SF samples of each KL grade- I, II, III, IV) as described below.

Estimation of NO

NO levels were measured by Griess reaction method; for this, SF samples were appropriately diluted, with an addition of 1% SA and 0.1% NEDD prepared in 5% phosphoric acid in 1:1 ratio. This mixture was incubated for 10 minutes at RT and an absorbance was measured at 540 nm in a plate reader (Biorad, Hercules, CA). Further calculations were performed using a standard curve made with linear concentrations of sodium nitrate.

Estimation of NN

Determination of NN levels were performed a using kit (Cayman, Ann Arbor, MI) by following the manufacturer's instructions. To measure nitrate, a mixture of 80 µl of SF sample (diluted in 1:5 ration) and 10 µl of the enzyme cofactor was added into each well; this was followed by an addition of 10 µl of nitrate reductase mixture. The reaction mixture was incubated at RT for 1 h and after the incubation, 50 µl of Greiss Reagent R1 was added. It was followed by an immediate addition of 50 µl of Greiss Reagent R2 and an incubation for 10 minutes at RT for color development. The absorbance was read at 540 nm. The wells with 200 µl assay buffer without any reagents were treated as blank. For the estimation of nitrite, the same procedure was followed by using 100 µl of SF (diluted in 1:5 ratio).

Nitrate-nitrite was calculated using following formula:

$$[\text{Nitrate+Nitrite}]\ (\mu M) = (A_{540}\text{-y intercept/slope}) \times (200\mu 1/\text{volumeofsampleused}(\mu 1)) \times$$

$$\text{dilution}[\text{Nitrite}](\mu M) = (\text{A540-y-intercept/slope}) \times (200\mu 1/\text{volumeofsampleused}(\mu 1)) \times$$

$$\text{dilution}[\text{Nitrate}](\mu M) = (\text{Nitrate+Nitrite}) - (\text{Nitrite})$$

Estimation of IL-1β

IL-1β levels in the SF samples were determined using ELISA kit (Abnova, Walnut, CA) according to the manufacturer's instructions. The optical density was measured at 450 nm using ELISA-microplate reader (Biorad, Hercules, CA).

4.5 Cell lines

Human monocyte cell line (THP1), human monocyte-like cells (U937) and hematopoietic stem cells (HSCs) were used in various cell-based studies of this thesis. THP1 and U937 cell line were purchased from National Centre for Cell Science (NCCS), Pune, India, while freshly isolated, certified hematopoietic stem cells (HSCs) from human bone marrow were procured from Nirav Biosolutions, Pune, India. All the cell types were maintained in Roswell Park Memorial Institute (RPMI)-1640 medium (HiMedia Laboratories, LLC, PA, USA) + 10% Fetal Bovine Serum (FBS) (HiMedia Laboratories, LLC, PA, USA) + 2 mmol/L L-glutamine + 1% penicillin + streptomycin (Sigma-Aldrich, St. Louis, USA), at 95% relative humidity and 5% CO_2 at 37°C.

4.6 To establish a functional involvement of SF in OA pathogenesis in terms of its potential to provide inflammatory microenvironment in the knee joint cavity (Publication 1, 2 and 3)

4.6.1 Estimation of NO release in SF challenge test

To test inflammatory potential of OA SFs, NO release in cell culture medium was measured after 48h of SF treatment. This test was performed on THP1 and U937 cell lines. In this assay, both cell types were seeded in 1×10^5cells/ml density; THP1 cells were induced with 10% SF (of culture medium), whereas SF concentration for U937 was 20% (of culture medium). SF concentration for cell induction was determined based on the outcomes of cell viability assay, which was performed as described (Koppikar, 2017) (data not shown). After 48h, the cell culture medium was collected and appropriately diluted with addition of 1% sulphanilic acid (SA) and 0.1% N, N-naphthylethylenediamine-dihydrochloride (NEDD), prepared in 5% phosphoric acid in 1:1 ratio and incubated for 10 mins at RT. After incubation, the absorbance was measured at 540 nm in a plate reader (Biorad, Hercules, CA). Further calculations were done from a standard curve made with linear concentrations of sodium nitrate (used as a standard) (Ingale et al., 2018). We used three SF samples of each KL grade (3 SFs X 4 KL grades = 12 SFs) for NO estimation. Equivalent SF controls in media without cells were maintained to blank the plate reader.

4.6.2 qRT-PCR analysis

After 48h of incubation, SFs induced U937 and THP1 cells were subjected to qRT-PCR analysis. Total RNA isolation from the cell pellet and qRT-PCR analysis was performed as described in the subsection 4.4.1 of the Methods. SF induction of THP1 cells was performed with KL grades-I, II, III and IV samples, while U937 cells were induced using SF of KL grade-II, III and IV. Gene expression analysis of the following pro-inflammatory markers was performed, where β-actin was kept as housekeeping gene.

Gene Name	TaqMan Gene Assay number
ACTB	Hs01060665_m1
MMP-1	Hs00899658_m1
MMP-13	Hs0023392_m1
VEGF-1	Hs00900055_m1

4.7 To assess an ability of SF to induce immune cell differentiation (publication 4 and 5)

4.7.1 Immunophenotyping of SF cells

To determine a fraction of macrophages in OA SFs, we performed immunophenotyping of SF cells from 12 fluid samples using a suitable antibody panel. For this, the collected SF samples were processed within 24h of fluid collection. At first, the samples were carefully examined for color, opacity, viscosity and any blood contamination. The samples with any sign of blood contamination were not considered suitable for this analysis. For SF cells isolation, the fluids were subjected to centrifugation at 1200 rpm at 4°C for 20 minutes. This step was repeated to remove any trace of SF from the obtained cell pellet, which was further washed with ice cold phosphate buffered saline (PBS) for 2 times at 1200 rpm at 4°C for 5 minutes. The resulting cell pellet was further aliquoted and used to stain with macrophages specific fluorescent labelled antibodies, such as CD14 (FITC, BioLegend, San Diego, US), CD86 (PECy7, BioLegend, San Diego, US) and CD163 (PerCP 5.5, BioLegend, San Diego, US) as per the manufacturer's instructions. The stained cell population was acquired using the gating strategy as showed in **Figure 7** (based on isotype control) and further analyzed on Attune Nxt Acoustic flow cytometer (Thermofisher Scientific, MA, USA). The flow cytometry data analysis was performed using Attune Nxt software (version 2.1) and the results are expressed in the percentage of positively stained cells.

4.7.2 *In vitro* cell differentiation assay

To assess a potential of OA SF to induce immune cell differentiation, *in vitro* cell differentiation assay was designed. In this assay, U937 cells were treated as

immune cell precursors and were exposed to different grades of OA SFs for 48h. After the incubation period, a status of the newly differentiated cells was analyzed using relevant flow cytometry markers. Briefly, the assay was as follows:

The cells were seeded with a density of 1 x 10^6 cells/ml in a 24 well plate and treated with 20 % (of culture medium) SF of different OA grades for 48h. We used 4 samples of each KL grade-II, III and IV for inducing the cells; thus, in total 12 OA SF samples were used. The cells treated with phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, St. Louis, USA) (dose – 100ng/ml) were used as a positive control and the untreated cells were used as a negative control. A safe dose of PMA to induce the cells was determined by cell viability assay, which was performed as described (Koppikar, 2017) (data not shown). After the incubation period, adherent cells were collected for FACS analysis. To harvest the cells without enzymatic digestion, they were incubated in 0.5 mM ethylene-diamine tetra-acetic acid (EDTA) in PBS for 15 minutes at 37°C and 5% CO_2. After 15 minutes, the cells were collected by repeated vigorous pipetting against the bottom of the 24 well plate.

The harvested cells were first blocked with human Fc blocking solution (Human TrueStain FcX, BioLegend, San Diego, US) in order to prevent any non-specific antibody binding and further stained with CD14 (FITC, Bio-Legend, San Diego, US), CD86 (PECy7, BioLegend, San Diego, US) and CD163 (PerCP 5.5, BioLegend, San Diego, US) as per the manufacturer's instructions. For each marker, percentage of positively stained cells were acquired using a BD FACSCanto II cell analyzer (BD Biosciences); for each experiment reaction, 10000 events were counted. The data analysis was per-formed with FACSDiva software (version 6.1.2). These flow cytometry experi-ments were performed in quadruplets and repeated for three times.

The *in vitro* cell differentiation assays were also performed on THP1 and HSCs with some modification to test another antibody panel of macrophages and mast cells respectively. In this module, both the cell types were treated with 10% SFs of different KL grades for 9 days. Cell culture media change was performed after every 3 days. On the 10^{th} day, the adhered cells were harvested and further stained by using the described protocol. THP1 cells were stained with human leukocyte antigen-DR isotype (HLA-DR) (PE-Cy5.5, Miltenyi Biotec, Bergisch Gladbach, Germany) and CD206 antibodies (PE, Miltenyi Biotec, Bergisch Gladbach, Germany), whereas HSCs were stained with Fc epsilon RI antibody (FcERI) (FITC, BioLegend, San Diego, CA, USA) as per the manufacturer's instructions. Here, for each cell surface marker, mean fluorescent intensity (MFI) was measured by Attune Nxt Acoustic flow cyto-meter (Thermofisher Sceintific, MA, USA). These flow cytometry experiments were performed in triplicate and repeated three times. For the SF treatment of THP1 and HSCs, 3 SF samples of each KL grade-I, II, III and IV (3 SFs X 4 KL grades = 12 SF samples) were used.

4.7.3 Functional analysis of the newly differentiated cells

Functional status of the newly differentiated cells from the *in vitro* cell assay on U937 was investigated in terms of the cytokine estimation produced by these cells. For this, qRT-PCR of the following cytokines was performed using the total RNA, isolated from the cell pellet of induced U937. The protocol for RNA isolation and qRT-PCR was followed as described in the subsection of 4.4.1 in the Methods section. mRNA amount of each gene was normalized to the amount of β-actin.

Gene Name	TaqMan Gene Assay number
ACTB	Hs01060665_m1
TNF-α	Hs00174128_m1
IL-6	Hs00985639_m1
IFN-γ	Hs00989291_m1
GMCSF	Hs00929873_m1
IL-4	Hs00174122_m1
IL-10	Hs00961622_m1

4.7.4 Proteome analysis of OA SF

To investigate a protein milieu of OA SF that could drive or regulate immune cell differentiation, we performed a proteome analysis of the fluids that were used in *in vitro* cell differentiation assay. Sixteen SFs were subjected to the analysis, wherein four samples of each KL grade were included (4 X 4 =16). At first, a protein depletion column was used to deplete abundantly present albumin and immunoglobulins from the samples as per the manufacturer's instruction (BioRad proeteoMinerprotein enrichment small-capacity kit; catalogue No. 1633006). Protein precipitation was attained by mixing each sample with the mixture of 100% trichoroacetic acid, 0.4% deoxycholate (TCA+DOC) in a 1:3 (v/v) ratio for 20 minutes at 4°C and later centrifuged for 15 minutes at 17000 rpm. The supernatant was discarded; the undisturbed pellet was re-suspended in 3 volumes (of the original sample volume) of RT 100% acetone, vortexed, incubated for 10 minutes at RT and centrifuged at 17000 rpm for 15 minutes. All the precipitates were air dried on ice for 10 minutes until no residual liquid was visible. The precipitated pellets were then suspended in 100µl of 7M urea, 2M thiourea, 100mM ammonium bicarbonate (ABC) solution (7/2 urea:thiourea buffer). After reduction and alkylation of cysteine bonds with 5 mM dithiothreitol and 20 mM chloroacetamide, respectively, for 1h at RT in the dark, each sample was digested for 4h in 1:50 (enzyme: protein) ratio using *Lysobacter enzymogenes* (Wako Pure Chemical Industries, Richmond, VA, USA). Solutions were diluted five times with 100 mM ABC and further digested overnight at RT with 1:50 dimethylated porcine trypsin (Sigma, Aldrich). The digested samples were then desalted using reversed phase

C18 StageTips. The samples were reconstituted in 0.5% trifluoro acetic acid (TFA) for the subsequent LC/MS/MS analysis, which was performed as described (Mets et al, 2019).

MS raw data were processed with the MaxQuant 1.4.0.8 software package (Cox and Mann, 2008). Methionine oxidation, asparagine/glutamine deamidation and protein N-terminal acetylation were defined as variable modifications, while cysteine carbamidomethylation was set as a fixed modification. Peptide search was performed against *in silico* trypsin digested (C-terminal cleavage after lysine/ arginine without proline restriction) UniProt (www.uniprot.org) *Homo sapiens* reference proteome database. First and main search MS mass tolerances were ±20 and ±4.5 ppm, respectively. MS/MS mass accuracy tolerance was ±20 ppm. Protein identifications were reported if ≥1 razor or unique peptides of ≥7 amino acids were identified. Transfer of peptide identifications (match between runs) based on accurate MS1 mass and RT was allowed. Protein quantification was reported if ≥1 peptide was quantified with ≥3 points. Label-free protein intensities were normalized using the MaxLFQ algorithm (Cox et al., 2014). Peptide-spectrum match and protein false discovery rate (FDR) were kept ≤1% using a target-decoy approach. All other parameters were as default.

4.8 Elucidation of molecular and cellular events in osteophytes and their linkage with OA pathology (Publication 4)

4.8.1 Osteophyte samples collection

Among the 32 study patients, osteophyte samples were collected from the six patients, who were undergoing knee replacement surgery (Institutional Ethics approval number – 282/T13). Demographic details of the patients from whom these samples were collected are given in Appendix as Table 4. The osteophytes were obtained from medial condyle of the tibia. We decided to focus particularly on the tibial osteophytes because, they are considered as one of the prominent features of OA (Hayeri et al., 2010) and further indicate the disease severity (Kishve and Motwani, 2020). Non-osteophytic tissue collected from epiphyseal trabecular bone from lateral condyle of tibia was used as a control. RNA-seq analysis of these samples (osteophytes as well as control specimens) were performed as explained below.

4.8.2 RNA isolation from osteophyte and control tissue samples

Approximately 50 mg of both the samples (osteophytes and control) were homogenized using liquid nitrogen and 500µl of Trizol reagent (Thermo Fisher Scientific Inc., CA, USA) in mortar and pestle. The homogenate was centrifuged at 12 000 x g, 10 min, at 4°C and the supernatant was transferred into a new 2.0 ml tube and incubated for 5 min at RT. 100µl of chloroform was added

into the sample, incubated it at RT for 2 min and centrifuged at 12 000 x g for 15 min at 4°C; the aqueous phase was transferred into a new 2.0 ml tube. Equal amount of freshly prepared 70% ethanol was added into the sample and transferred into Rneasy Mini spin column for further RNA isolation as per the manufacturer's instructions (Qiagen, Valencia CA, USA). Quality of total RNA was evaluated using Agilent 2100 Bioanalyzer with RNA 6000 Nano Kit (Agilent Technologies Inc., CA, USA) and the quantity was estimated using Qubit 2.0 fluorometer with RNA HS Assay Kit (Thermo Fisher Scientific Inc., CA, USA). The average RNA integrity number (RIN) of samples was between 2.8 and 7.7. The samples were stored at -80°C until further processing.

4.8.3 RNA-seq analysis of osteophytes

50 ng of the total RNA of osteophyte and control tissue was amplified by applying Ovation RNA-Seq System V2 (NuGen, Emeryville, CA, USA) after which the resulting cDNAs were used to prepare DNA fragment library with SOLiD System chemistry and barcode adaptors (Thermo Fisher Scientific Inc., CA, USA). Prior to the sequencing, all 12 libraries were labelled with different barcodes and were pooled together in equal amounts. Sequencing was performed using SOLiD 5500W platform and fragment sequencing chemistry (Thermo Fisher Scientific Inc., CA, USA).

Raw reads (75 bp) were color-space mapped to the human genome hg19 reference using Maxmapper algorithm implemented in the Lifescope software (Thermo Fisher Scientific Inc., CA, USA). Mapping to multiple locations was permitted. The quality threshold was set to 10, giving the mapping confidence more than 90. Reads with the score less than 10 were filtered out. Average mapping quality was 30. Analysis of the RNA content and gene-based annotation was done with whole transcriptome work-flow. Raw sequencing data with appropriate experimental information is available in the NCBI Gene-Expression Omnibus (GEO) repository under the accession number GSE66511.

After quality control of the samples, to perform differential gene expression analysis, non-normalized raw counts were used for the EdgeR package. EdgeR is flexible tool for RNAseq data analysis to find differentially expressed genes. It performs model-based scale normalization, estimates dispersions and applies negative binomial model. Further, it implements negative binomial model fitting followed by testing procedures for determining differential expression (Robinson et al., 2010).

4.8.4 Transcriptome outcomes validation by immunohistochemistry (IHC) and qRT-PCR:

4.8.4.1 IHC of osteophyte and control tissue samples

Accounting the transcriptomics outcomes, mast cells presence in the osteophytes was confirmed by IHC of the study samples. For this, the osteophyte and control tissue specimens were decalcified with Sakura TDE 30 Decalcifier System and

were embedded in paraffin after fixation in formalin. The 5 µm sections of the specimens were cut, deparaffinized and were treated with 0.9% H_2O_2 to inactivate endogenous peroxidase. The sections were further treated with Dako REAL Antibody Diluent (S2022; Dako Denmark A/S, Glostrup, Denmark) to block non-specific binding. After blocking, the sections were incubated with the mouse monoclonal antibody to Tryptase Alpha/Beta1 (TPSAB1) (MA5-11711, Thermofisher) and rabbit polyclonal antibody to FcERI (ab229889, Abcam) to stain mast cells overnight at 4°C. Primary antibody dilution was 1:200. Visualization of the primary antibody was performed using the commercial kit "Dako REAL EnVision DetectionSystem, Peroxidase/ DAB+, Rabbit/Mouse" (K5007; Dako Denmark A/S, Glostrup, Denmark). Washing steps in-between were performed using PBS, which contained 0.07% of Tween 20 as the detergent. Toluidine blue (Applichem, Darmstadt, Germany) was used for background staining. No immune staining was noted in negative controls, where the primary antibody was omitted. IHC images were obtained with Zeiss LSM-510 Confocal Laser-scanning microscope. Ordinal method was used to calculate mast cells staining frequency as described (Meyerholz and Beck, 2018). This method is a semiquantitative method, that reflect cellular staining frequency or intensity. Using this cellular frequency, we estimated the mast cells staining incidence (%) in the control tissues and the osteophyte samples.

4.8.4.2 qRT-PCR validation

The presence of key up-regulated, revealed during transcriptome analysis, was confirmed by qRT-PCR, which was performed as described in 4.4.1 of the Methods section. Due to unavailability of control tissue RNA, the qRT-PCR validation was limited to confirm the expression of the following key upregulated genes of the transcriptomics data. The amount of the expressed genes was normalized to β-actin.

Gene Name	TaqMan Gene Assay number
ACTB	Hs01060665_m1
CPA3	Hs00157019_m1
CMA1	Hs00156558_m1
TPSAB1	Hs02576518_gH
MMP1	Hs00899658_m1
MMP3	Hs00968305_m1
MMP13	Hs0023392_m1

5. STATISTICAL ANALYSIS

Synovial biopsies gene expression study, cell assays for NO determination and qRT-PCR analysis of SF challenge test were performed in triplicates and the data is presented as mean ± SD. Inter-grade statistical significance in these experiments was determined by One Way ANOVA followed by a Kruskal–Wallis test and Tukey's test of significance using R software version 3.3.0.

Data of *In vitro* cell differentiation assay on U937 cells was analyzed using FACSDiva software (version 6.1.2); data analysis of the cell differentiation assay on THP1 and HSCs and immunotyping of SF cells was performed using attune Nxt software (version 2.1). Inter-grade statistical significance was performed by one-way ANOVA followed by Bonferroni's multiple comparison test using GraphPad Prism 5 Program (San Diego, CA, USA).

Differential transcriptome between osteophytes and non-osteophytic control tissue was determined by applying group-wise comparisons, where negative binomial fitting was followed by an exact test. False discovery rate (FDR) adjustment was used for multiple testing corrections. FDR threshold of 0.05 was applied for statistical significance. The data analysis of qRT-PCR validation of the transcriptome and IHC study was carried out using GraphPad Prism 5 Software (San Diego, CA, USA) using one-way ANOVA followed by Bonferroni's test for multiple comparisons. In case of SF proteome analysis, differentially expressed proteins in the fluid samples of KL grade-II, III and IV were detected against the proteins expressed in SFs of KL grade-I (used, as a control group) by performing a paired t-test.

In all the experiments, P values < 0.05 were considered significant.

6. RESULTS

6.1 To outline and compare a variation pattern of key inflammatory mediators at different stages of natural OA progression (Publication 1 and 3)

6.1.1 Synovium gene expression study

The highest expression of IL-1β and IL-15 was found in synovium biopsies of KL grade-I. Thereafter, there was a decline in the expressions of both the cytokines except the biopsies from KL grade-IV, wherein a trivial increase was noted. In case of IL-1β expression, a significant difference was noted between KL grade-I and II biopsies ($P = 7.70 \times 10^{-3}$; $P < 0.05$). IL-15 also showed a marked difference for KL grade-I and III biopsies ($P = 3.66 \times 10^{-2}$; $P < 0.05$) (**Figure 4 A**, **B**). However, no significant difference was observed among the biopsies of other KL grades. PGE2 and NGF did not show any expression trend and the highest mRNA levels were found in KL grade-II and grade-IV biopsies, respectively. Both the markers did not reveal any significant observation for inter-grade comparison (**Figure 4 C**, **D**).

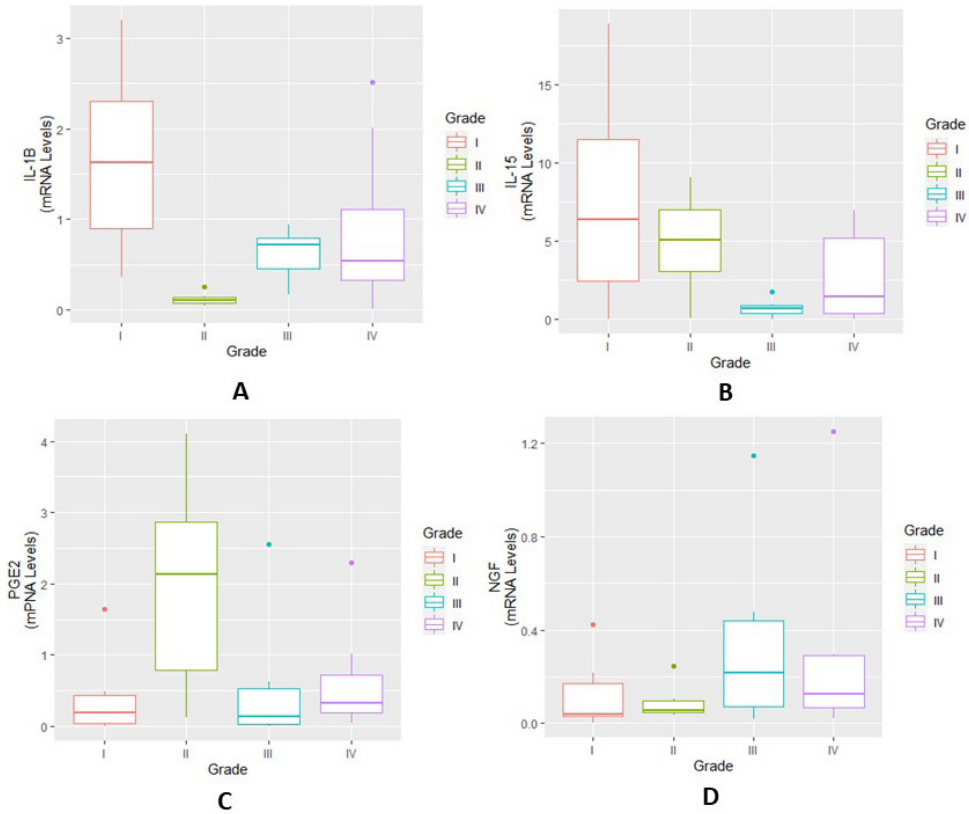


Figure 4: Synovial biopsy gene expression study – expression of pro-inflammatory markers was estimated in synovial biopsy samples spanning different grades of OA patients (n=26); **A**) a grade-wise expression trend of IL-1β; peak expression was found in the samples collected from KL grade-I OA patients and the lowest expression was noted in KL grade-II samples. A significant difference was noted between KL grade-I and II (P<0.05); **B**) expression trend of IL-15 revealed a grade-wise decline; the highest expression was noted in KL grade-I biopsies, while a trivial increase was noted in KL grade-IV samples; a significant difference was noted between KL grade-I and III (P<0.05); **C**) a grade-wise expression pattern of PGE2 with the highest expression in KL grade-II biopsies; inter-grade comparison for this marker did not reveal any significant change among all the KL grades; **D**) expression pattern of NGF, wherein the highest expression was seen in KL grade-IV biopsies; no significant difference was noted among the grades for inter-grade comparison

6.1.2 Estimation of pro-inflammatory mediators in SF samples

The highest IL-1β levels were estimated in KL grade-III SF samples, followed by SFs of KL grade-II. IL-1β levels in KL grade-I and IV were comparable. There was no statistically significant difference in the levels, when intergrade comparison was performed (**Appendix Table 5**).

The highest NO release was estimated in the KL grade-II SF samples; in fact, all the SF samples from KL grade-II, III and IV showed comparable NO levels. The lowest NO levels were found in early grade-OA (KL grade-I) samples. Similar to IL-1β, intergrade comparison for NO did not reveal any notable difference (**Appendix Table 5**).

The highest NN levels were detected in KL grade-IV SF samples, followed by KL grade-II samples. The SF samples of KL grade-I and III showed comparable NN levels. During intergrade comparison, a significant difference was noted in KL grade-I and IV ($P < 0.01$) as well as KL grade-III and IV samples ($P < 0.05$) (**Appendix Table 5**).

Table 2: a representative data set of SF samples with their KL grades, used for estimation of the pro-inflammatory factors; a complete data set with statistical analysis is presented as **Appendix Table 5**

Sr. No	Patients	KL grade	IL-1β (pg/ml)	NO (μM/ml)	Nitrate/nitrite (μM)
1	Patient 1	4	23.64 ± 1.8	69.11 ± 15.4	24.65 ± 0.7
2	Patient 2	4	22.27 ± 7.7	62.36 ± 0.6	77.66 ± 2.6
3	Patient 3	3	603.03 ± 12.7	42.35 ± 7.7	42.98 ± 1.0
4	Patient 4	2	2184.85 ± 10.5	11.47 ± 1.4	66.54 ± 1.9
5	Patient 5	2	642.42 ± 13.8	80.00 ± 7.6	82.68 ± 1.9
6	Patient 6	1	47.27 ± 4.5	14.70 ± 0.6	23.56 ± 0.0
Normal values			9.30–13.10 (Karan et al., 2003)	–	

6.2 To establish a functional involvement of SF in OA pathogenesis in terms of its potential to provide inflammatory microenvironment in the knee joint cavity (Publication 2 and 3)

6.2.1 NO estimation in SF challenge test

NO estimation was performed in the cell culture media after 48h of SF treatment; this experiment was performed on two cell lines – THP1 and U937.

THP1

THP1 cells after SF treatment showed a grade wise increase and maximum NO release was observed in the cells treated with SFs of KL grade-II and III as compared to control cells. A grade wise pattern of NO estimation and statistical significance is demonstrated in **Figure 5A**.

U937

In U937 cells, the highest NO release was estimated after the treatment of SF of KL grade-I; these NO levels were significantly higher as compared to control, PMA as well as SF induction with KL grade-II and III. Treatment with SF of KL grade-II and III also revealed a significantly increased NO in comparison with control, but not with PMA (**Figure 6A**). Intergrade statistical significance in NO levels is showed in **Figure 6A**.

6.2.2 qRT-PCR analysis of inflammatory markers:

Inflammatory response by SF induced THP1 and U937 cells was measured in the form of mRNA abundance of pro-inflammatory markers like, MMP-1, MMP-13 and VEGF-1.

THP1

The highest MMP-1 expression was noted after the SF treatment of KL grade-II; a grade-wise decline was observed thereafter. During inter-grade comparison, the cells treated with SF KL grade-I showed a significant decrease in MMP-1 expression, when compared to KL grade-II ($P = 1.93 \times 10^{-14}$; $P < 0.001$) and KL grade-III ($P = 5.48 \times 10^{-6}$; $P < 0.001$). A significant difference in the expression level was also noted between the cells treated with SF of KL grade-II and III ($P = 2.80 \times 10^{-14}$; $P < 0.001$), KL grade-II and IV ($P = 1.92 \times 10^{-14}$; $P < 0.001$) and KL grade-III and IV ($P = 1.37 \times 10^{-6}$; $P < 0.001$). MMP-1 expression between SF treatment by KL grade-I and IV was insignificant ($P = 0.901$) (**Figure 5B**).

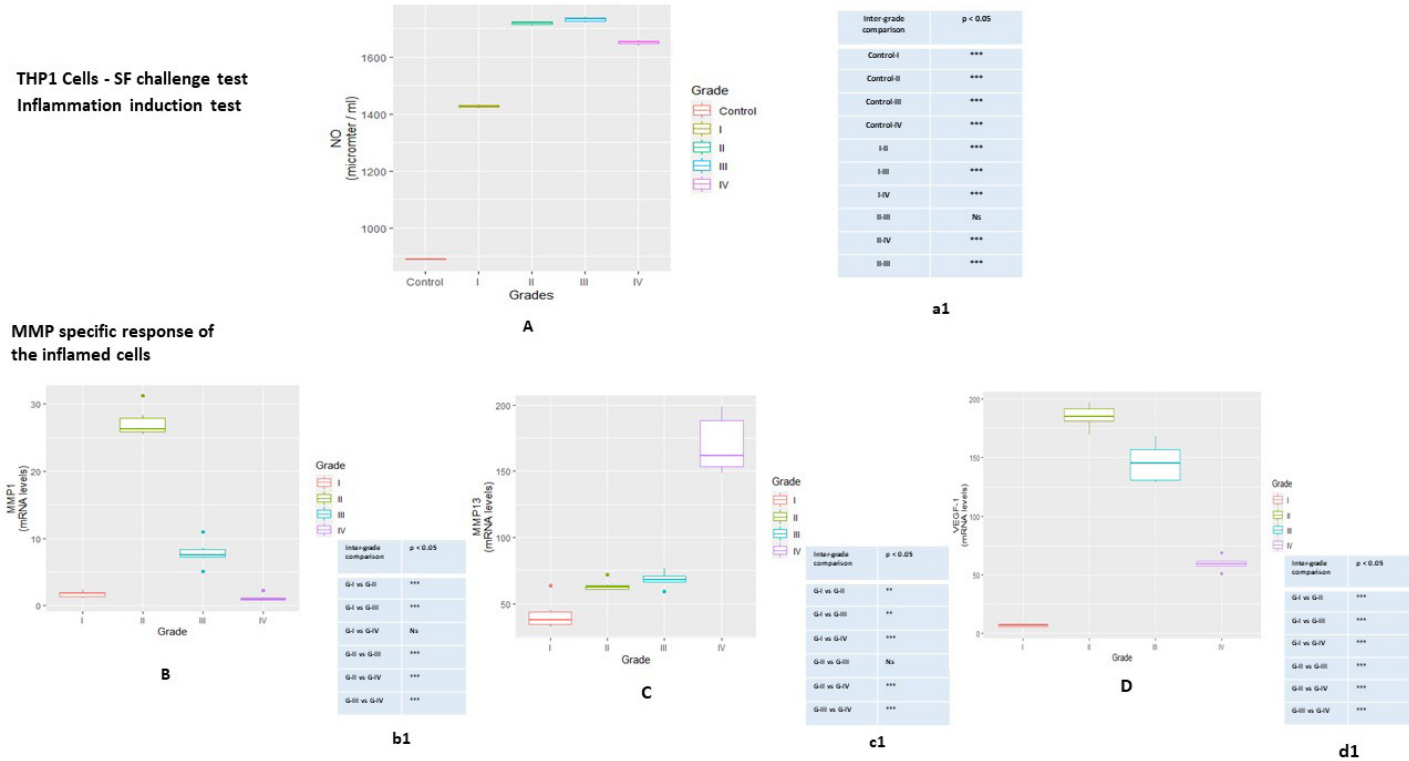


Figure 5: SF challenge test on THP1 cells – THP1 cells were treated with 10% SFs (of culture media) of progressive OA grades for 48hrs in order to induce inflammation. **A**) NO release estimated after 48hrs of SF treatment; NO significance revealed during inter-grade comparison is showed in table **a1**; **qRT-PCR analysis of inflammatory markers – B**) MMP-1: the highest rise in the expression in the cells treated with SFs from KL grade-II; **C**) MMP-13: a grade-wise elevation was seen in MMP-13 expression; the maximum up-regulation was found in the cells treated with SFs from KL grade-IV; **D**) VEGF-1: the highest estimation of VEGF was in the cells treated with KL grade-II SFs that was followed by a grade-wise decline in the expression; the lowest expression was seen in the cells treated with SFs from KL grade-I.
Inter-grade difference for MMP-1, MMP-13 and VEGF-1 on the cells is showed in tables **b1**, **c1** and **d1**, respectively.
*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

MMP-13 expression in SF treated THP1 showed a grade-wise increase; the highest level of this gene was found in the cells treated with KL grade-IV. In inter-grade comparison, a significant difference in MMP-13 expression was found for the SF treatment with all KL grades except the treatment between SF of KL grade-II and grade-III (P = 0.923). The difference in the expression revealed in the inter-grade comparison was demonstrated as - KL grade-I vs. II ($P = 3.91 \times 10^{-2}$; $P < 0.05$), KL grade-I vs. III ($P = 1.01 \times 10^{-2}$; $P < 0.05$), KL grade-I vs. IV ($P = 1.19 \times 10^{-12}$; $P < 0.001$), KL grade-II vs. IV ($P = 3.76 \times 10^{-11}$; $P < 0.001$), KL grade-III vs. IV ($P = 8.45 \times 10^{-11}$; $P < 0.001$) (**Figure 5C**).

VEGF-1 expression trend was similar to MMP-1 expression; this means, maximum expression of this gene was noted in THP1 cells treated with SFs of KL grade II, which was followed by a grade-wise decline. Inter-grade comparison for VEGF-1 showed a marked difference in the SF treatment of KL grade-I vs. II ($P = 1.92 \times 10^{-14}$; $P < 0.001$), KL grade-I vs. III ($P = 2.19 \times 10^{-14}$; $P < 0.001$), KL grade-I vs. IV ($P = 8.58 \times 10^{-8}$; $P < 0.001$), KL grade-II vs. III ($P = 8.53 \times 10^{-6}$; $P < 0.001$), KL grade-II vs. IV ($P = 3.44 \times 10^{-14}$; $P < 0.001$) and KL grade-III vs. IV ($P = 1.89 \times 10^{-11}$; $P < 0.001$) (**Figure 5D**).

U937

MMP-1 showed the highest expression in the cells when induced with SF of KL grade-III; it was significantly higher when compared to UC ($P = 7.72 \times 10^{-8}$; $P < 0.001$). SF treatment with KL grade-II and IV were also significantly elevated MMP-1 as compared to UC (KL-II vs UC – $P = 4.13 \times 10^{-4}$ and KL-IV vs UC – $P = 8.07 \times 10^{-5}$; $P < 0.001$) (**Figure 6B**).

MMP-13 expressions after induction with SF from KL grade-II, III and IV were significantly higher when compared to UC (KL-II vs UC – $P = 8.55 \times 10^{-7}$, KL-III vs UC – $P = 1.69 \times 10^{-7}$, KL-IV vs UC – $P = 4.19 \times 10^{-6}$; $P < 0.001$). SF induction with KL grade-II and III revealed a comparable expression level of MMP-13 (**Figure 6C**).

VEGF-1 showed an expression peak in the cells induced with SF of KL grade-III ($P = 1.86 \times 10^{-6}$; $P < 0.001$); SF treatment with KL grade-IV also caused a marginal increase in the expression when compared to UC ($P = 5.04 \times 10^{-3}$; $P < 0.05$). However, SF treatment with KL grade-II did not cause any significant change in VEGF-1 level (P = 0.928) (**Figure 6D**).

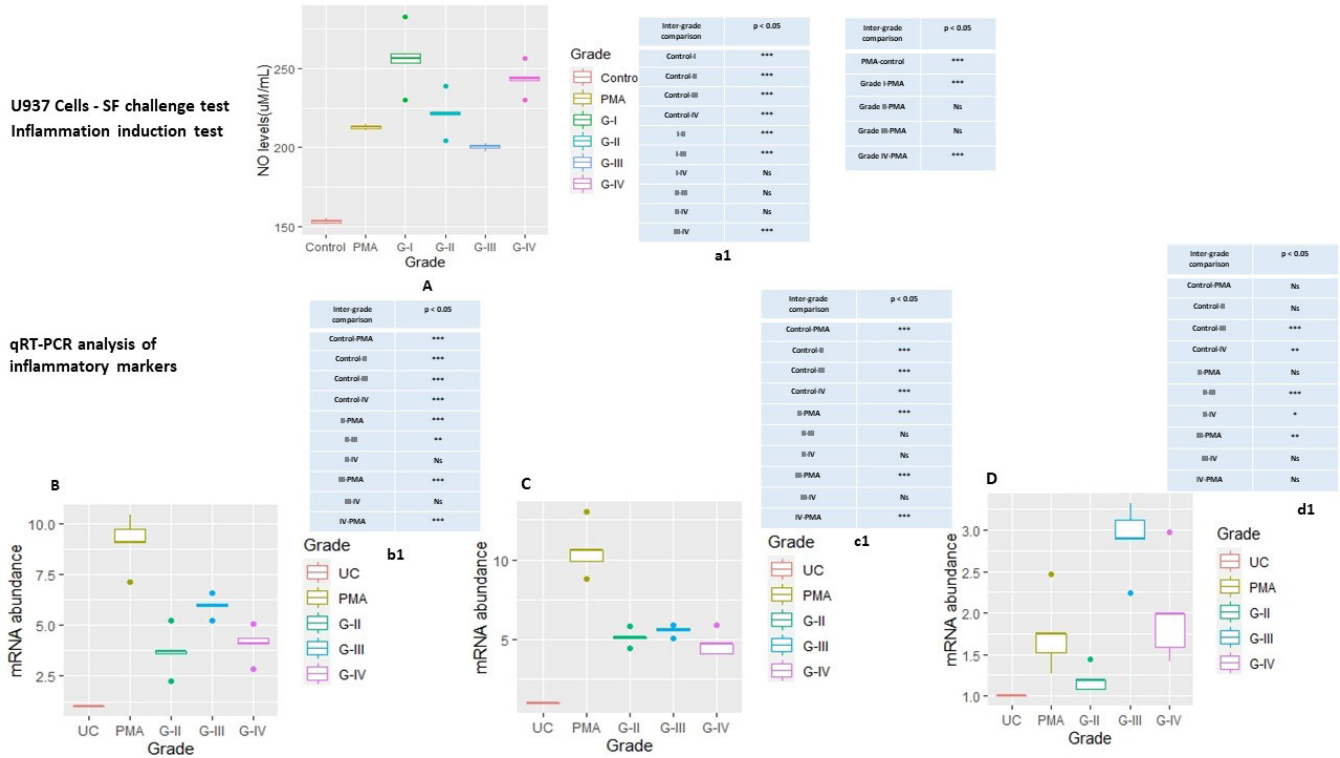


Figure 6: SF challenge test on U937 cells – U937 cells were treated with 20% SFs of progressive OA grades for 48h to induce inflammation. **A**) NO release trend measured after 48h of SF induction with KL grade-I, II, III and IV. Intergrade comparison with statistical significance is listed in table a1; **qRT-PCR analysis of inflammatory markers – B**) MMP-1: the highest expression was found in the cells, treated with SF of KL grade-III; SF treatment with KL grade-II and IV also showed a significant increase in MMP-1; **C**) MMP-13: revealed a similar expression trend of MMP-1; significantly elevated levels were observed after induction with SFs of KL grade-II, III and IV; **D**) VEGF-1: a peak expression was found in the cells treated with SF of KL grade-III; SF treatment with KL grade-IV also showed a marginal increase in the expression. Inter-grade difference for MMP-1, MMP-13 and VEGF-1 on the cells is showed in tables **b1**, **c1** and **d1**, respectively. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

6.3 To assess an ability of SF to induce immune cell differentiation (Publication 4 and 5)

6.3.1 Immunophenotyping of SF cells to estimate immune cell percentage (Publication 5)

Immunophenotyping of the cells in freshly collected SF samples was performed focusing on estimation of monocyte/macrophages. Thus, antibody panel selected for these experiments included cell surface markers like, CD14, CD86 and CD163, which denote monocyte/macrophages and their subsets. In these experiments four SF samples of each KL grade-II, III and IV were included (n = 12). Percentage of CD14+ monocytes/macrophages was highest in KL grade-II samples, followed by a significant decline in KL grade-III and IV (**Figure 7C**). Percentage of CD86+ and CD163+ cells also showed a grade-wise decline; however, the percentages of these cells were minimal (**Figure 7 D and E**).

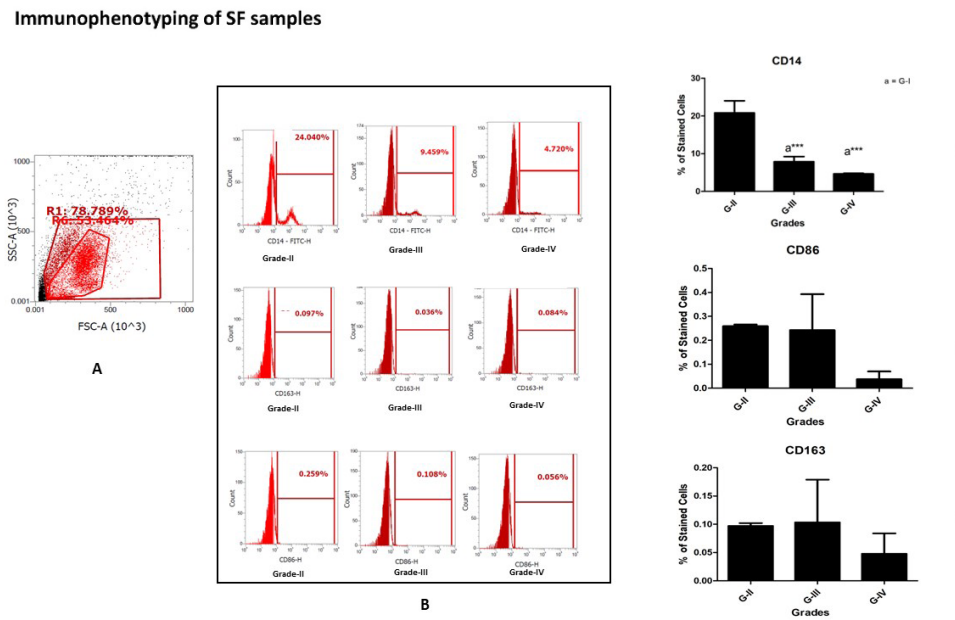


Figure 7: Immunophenotyping of SF cells to estimate monocyte/macrophage percentage – Freshly collected twelve SF samples were subjected to immunophenotyping to estimate monocyte/macrophages percentage; **A**) denotes the gating strategy established on isotype control for the selection of cell population; **B**) representative histograms of macrophages and their subsets analysis in OA SFs from KL grade-II, III and IV; for the identification of macrophages and their subsets, the selected antibody panel included cell surface markers like CD14 (pan-macrophage marker), while CD86 and CD163 represent M1 and M2 phenotype of macrophages, respectively. Values in the quadrant represent the percentage of positive cells; **C**, **D** and **E** are the bar-graphs, representing a grade-wise staining pattern of CD14+, CD86+ and CD163+ cells in SF samples; for plotting these graphs, the average of each cell surface marker obtained from four SF samples of each KL grade was used (n=12)
[a] statistical significance in comparison to G-II
$*P < 0.05$, $**P < 0.01$, $***P < 0.001$

6.3.2 *In vitro* cell differentiation assay

6.3.2.1 U937 cells (Publication 5)

Status of newly differentiated cells after 48h of SF induction was evaluated with macrophages specific antibody panel including CD14, CD86 and CD163. Percentage of CD14+ cells showed a grade-wise increase; this means, the highest percentage of CD14+ cells was seen after the SF induction with KL grade-IV. Inter-grade comparison for CD14+ cell phenotype was insignificant on statistical scale (**Figure 8B**). CD86+ cells exhibited a differentiation pattern similar to CD14+; thus, a grade-wise elevation was noted in CD86+ phenotype. Induc-

tion with SF of KL grade-III and IV showed a marked increase in the percentage of CD86+ cells ($P < 0.01$) (**Figure 8C**). Unlike CD14 and CD86, percentage of CD163+ cells were dramatically increased after the induction with KL grade-II ($P< 0.001$). This increase was followed by a decline in the percentage of CD163+ after the treatment of KL grade-III and was comparable to the percentage of CD163+ cells in control. KL grade-IV SF treatment showed some increase in CD163+ cells (**Figure 8D**).

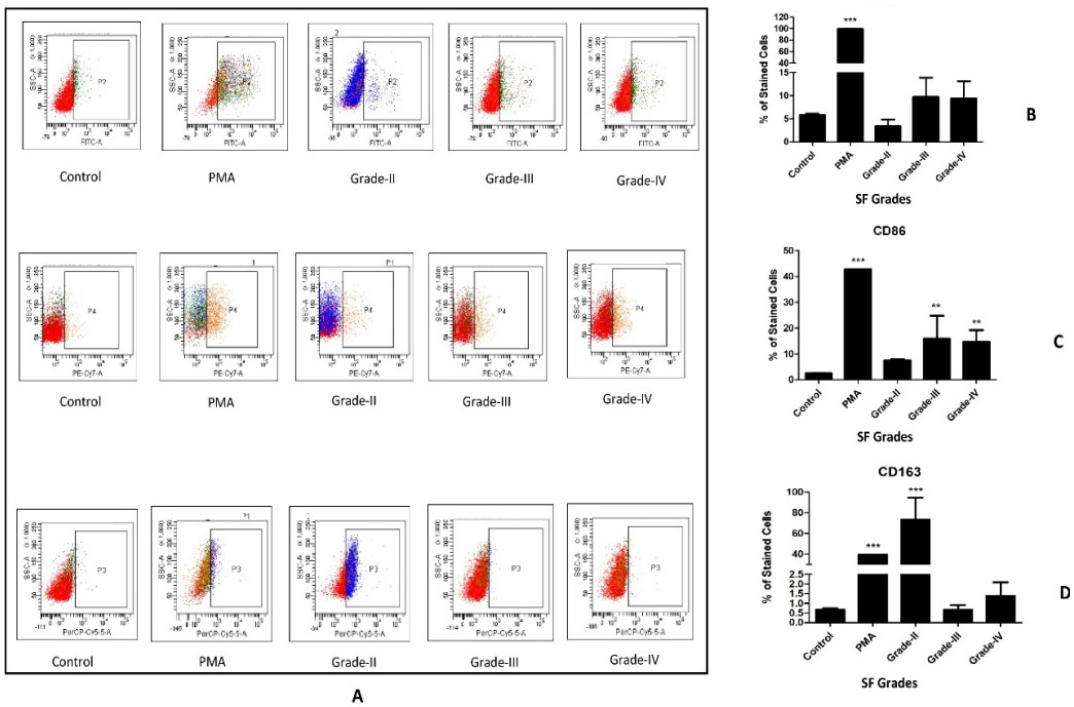


Figure 8: *In vitro* cell differentiation assay on U937 cells – U937 cells were induced using SF of KL grade-II, III and IV for 48h and status of the newly differentiated cells were evaluated using macrophages specific cell surface markers – CD14, CD86 and CD163; **A**) representative scatterplots of macrophages and their subtypes; CD14 is a marker for M0 macrophages, while CD86 and CD163 represent M1 and M2 subtype, respectively; the gating strategy of cell population selection was based on isotype control; **B**, **C** and **D** demonstrate a grade-wise staining trend for CD14+, CD86+ and CD163+ cells, respectively; for these graphs, we used average percentage of each cell surface marker calculated from three sets of experiment, wherein four SF samples of each KL grade were used in each set of experiment (n=12)
*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, as compared to control

6.3.2.2 THP1 and HSCs (Publication 4)

For this set of experiment, SF induction was performed for 9 days. 10^{th} day flow cytometry analysis of THP1 showed a clear and prominent differentiation into HLA-DR+ and CD206+ cells, when compared to untreated control (UC) and PMA induced cells (**Figure 9a**, **b**). Both the antibodies revealed a similar staining pattern, wherein the highest number of differentiated cells was noted after SF induction with KL- grade III fluid samples (**Figure 9a**, **b**).

10^{th} day flow cytometry analysis of HSCs revealed a significant differentiation into FcERI+ cells after the treatment of SF samples from KL-grade III as compared to UC, PMA (Figure 10c). The staining pattern of FcERI was also similar to HLA-DR and CD206 staining in THP1 cells (**Figure 9a1**, **9b1**, **9c1**).

Collectively, the outcomes of the *in vitro* cell differentiation assay performed on various cell types showed that OA SF can drive a clear and significant immune cells differentiation like, monocytes into macrophages and HSCs into mast cells.

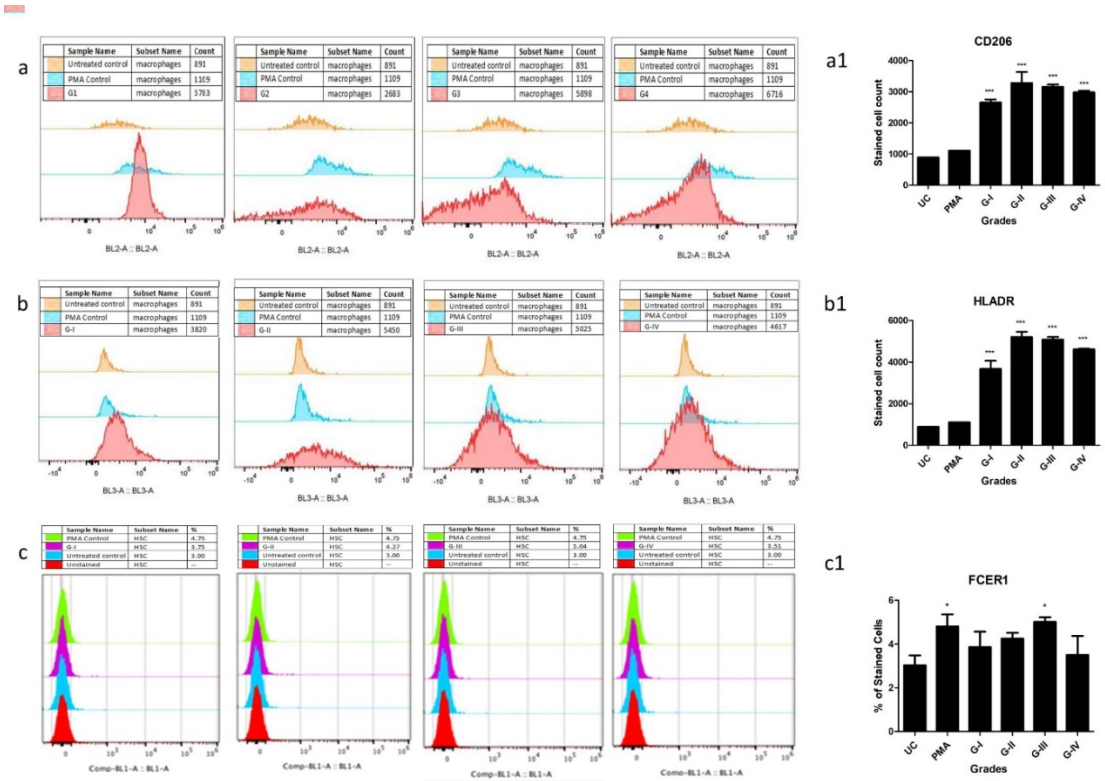


Figure 9: *In vitro* cell differentiation assay on THP1 and HSCs – THP1 and HSCs were induced with 10% (of culture medium) SF of KL grade I to IV for 9 days; **a**, **b** and **c** are the representative overlaid histograms of CD206, HLA-DR and FcERI, respectively, from the 10^{th} day flow cytometry analysis; CD206 and HLA-DR were analyzed in induced THP1, while FcERI was analyzed in induced HSCs; for each cell surface marker, number of positively stained cells obtained after the SF induction from different KL grades is showed in the table on the top of the respective histograms; the gating strategy was based on isotype control; **a1**, **b1** and **c1** represent a grade-wise staining trend obtained for CD206, HLA-DR and FcERI, respectively; for these bar-graphs, average value of each cell surface marker estimated from three SF samples of each KL grade was used (n=12); the experiment was performed in triplicates and repeated for three times

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, as compared to UC

6.3.2.3 Functional status analysis of the newly differentiated cells (Publication 5)

This experiment was performed on the SF induced U937 after 48h. In the functional analysis, gene expression of marker genes was measured by RT-PCR in the newly differentiated cells. Expression of IL-6 was significantly higher in the differentiated cells, which were induced by SF of KL grade-III, when compared to untreated cells ($P < 0.01$). TNF-α and IFN-γ expressions were noted significantly higher in the cells induced with SF of KL grade-IV ($P < 0.05$ and $P < 0.01$, respectively). On the other hand, IL-4 and IL-10 showed a similar expression pattern with the peak in the differentiated cells, which were induced by SF of KL grade-II ($P < 0.01$). among the selected cytokines panel, IL-6, TNF-α, IFN-γ and GMCSF are pro-inflammatory in nature (M1 specific secretions), while IL-4 and IL-10 are known to be produced by M2 type of macrophages. All the cytokine estimation trends were well correlated with their flow cytometry analysis (**Figure 8** and **Figure 10**).

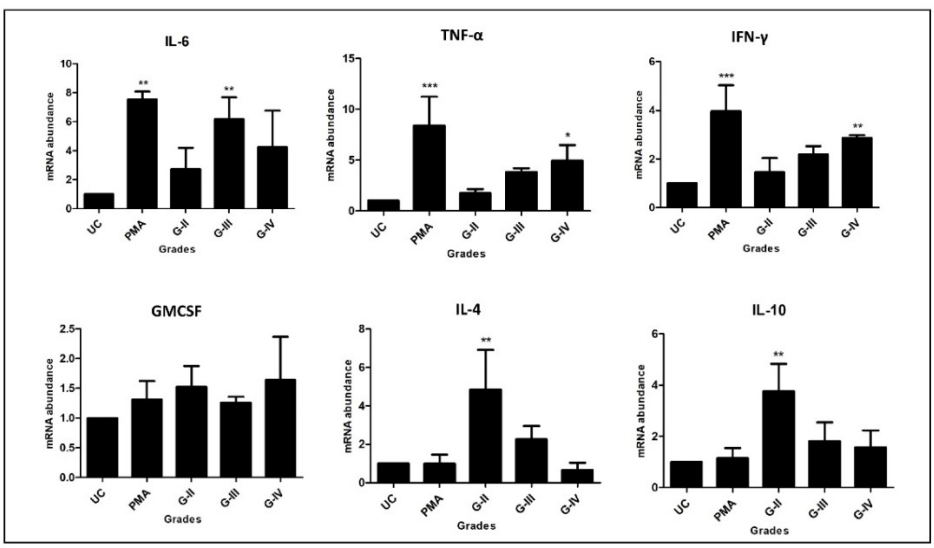


Figure 10: Functional status of the newly differentiated cells from *in vitro* U937 cells – The newly differentiated cells were investigated for their cytokine production to confirm their functional viability; for this, mRNA expression amount of each cytokine gene was estimated using qRT-PCR and normalized against ACTB, a house keeping gene; in the cytokines panel, IL-6, TNF-α, IFN-γ and GMCSF are M1 macrophage specific, and IL-4 and IL-10 are M2 specific cytokines; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, as compared to UC.

6.3.3 Assessment of a proteome in SF samples of progressive OA grades (Publication 4 and 5)

Proteome analysis of SF samples from different KL grades was performed to find out the protein-complement of these fluids, which was possibly responsible for driving immune cells differentiation as was evident in the *in vitro* cell differentiation assays. For this, the fluid samples those were used in *in vitro* assays were used and the expressed proteins were identified against UniProt proteome database. (Accession date –13 November 2020; The UniProt Consortium, 2021). As KL grade-I denotes doubtful OA condition, the proteins expressed in KL grade-I SF samples were used as a control group. In total, 799 differentially up-regulated proteins were found as compared to the control group. **Figure 11** depicts protein frequency against their log of fold-change (logFC) values in a comparative analysis of SF samples of different grades. Thus, in a comparative analysis of KL grade-II vs grade-I, approximately 100 proteins revealed more than 25-fold increase in their expression, while around 25 proteins were significantly down-regulated with a fold change between -25 to -50. Approximately, 200 proteins were remarkably up-regulated with more than 25-folds change in their expression in a comparative analysis of KL grade-III vs grade-I and KL grade-IV vs grade-I. Briefly, terminal grade SF samples (KL grade-III and IV) showed a significant variation in the protein make-up than early grade fluid samples (KL grade-II). Pro-inflammatory S100 proteins, histones, mitogen-activated protein kinase (MAPK) family, light chain immunoglobulins, and mast cells de-granulation proteases such as carboxypeptidase and cathepsins were the major subset of proteins, which were differentially up-regulated (**Appendix Table 2**). A grade-wise presentation and Reactome pathway analysis of gene-ontology processes of these protein subsets are presented in **Figure 12**. The Reactome pathway analysis revealed a significant up-regulation of the pathways like, innate immune system, Fc-gamma receptor (FcGR)-dependent phagocytosis coupled with a marked accumulation of light chain immunoglobulins were a clear implication of immune system involvement in OA pathology. Particularly, high expression of S100A8, S100A9, S00A11 and S100A12, the members from S100 protein family in all grades of SF samples underlined an activated status of macrophages and neutrophils and inflammation [**Figure 12 (3)**]. Also, differentially up-regulated proteases such as carboxypeptidase, carboxypeptidase Q and cathepsins (L1, D, B and G) were the signatures of prominent mast cell activity.

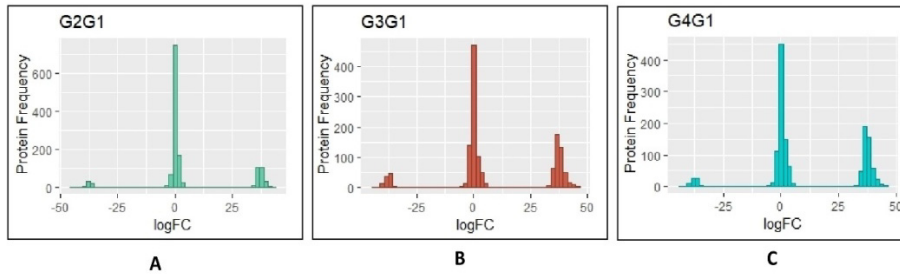
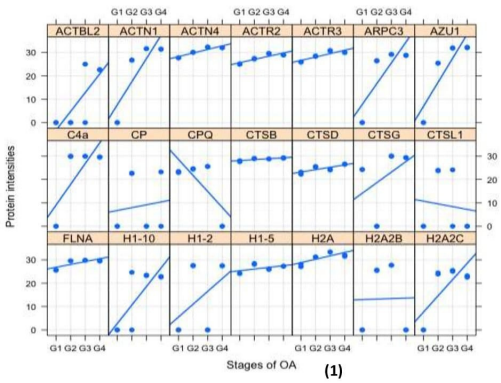
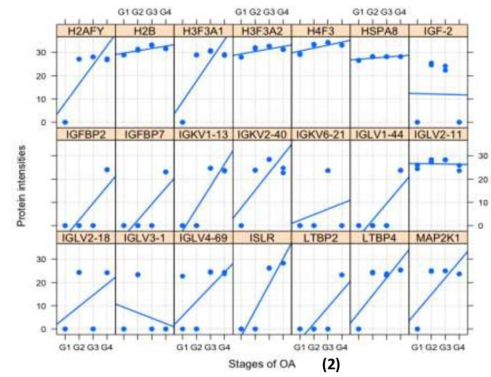
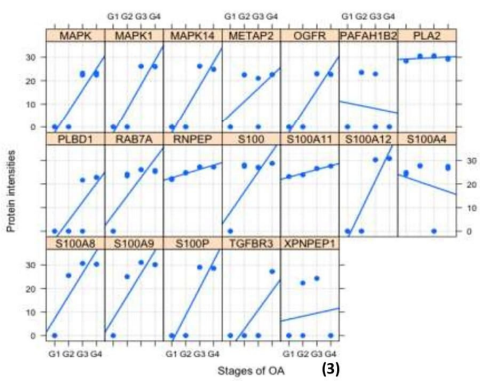


Figure 11: an overview of proteome analysis of SF samples – sixteen SF samples, four fluid samples from each KL grade-I, II, III and IV, were subjected to LC/MS/MS analysis to determine the proteome in these samples; in total, 799 proteins were found differentially expressed; **A**, **B** and **C** are the frequency histograms, which represent proteins' frequency against their logFC values, revealed during a comparative analysis of KL grade-II vs grade-I, KL grade-III vs grade-I and KL grade-IV vs grade-I, respectively; during the analysis, differentially expressed proteins were identified against the reference protein database of UniProt Homo sapiens proteome







Reactome Key Pathways	P-value	FDR
Innate Immune System	2E-15	1E-12
RHO GTPase Effectors	3.13E-14	7.86E-12
FCGR3A-mediated phagocytosis	1.47E-13	1.32E-11
Leishmania phagocytosis	1.47E-13	1.32E-11
Parasite infection	1.47E-13	1.32E-11
Regulation of actin dynamics for phagocytic cup formation	1.59E-13	1.32E-11
Fcgamma receptor (FCGR) dependent phagocytosis	1.92E-12	1.36E-10
RNA Polymerase I Promoter Opening	2.8E-12	1.74E-10
Signaling by Rho GTPases	9.25E-12	5.09E-10
Immune System	4.98E-11	2.49E-9
Cellular Senescence	5.55E-11	2.5E-9
Oxidative Stress Induced Senescence	6.82E-11	2.8E-9
Leishmania infection	1.28E-10	4.86E-9
Senescence-Associated Secretory Phenotype (SASP)	2.31E-10	8.08E-9
DNA methylation	2.57E-10	8.47E-9

(4)

Figure 12: Proteome analysis of SF samples – Lattice plots **(1)**, **(2)**, **(3)** represent a grade-wise picture of different protein subsets, which were differentially expressed in OA SFs; differential protein expressions were determined by comparing the logFC

value of each protein found in KL grade I SF samples; these proteins contain immune cell regulatory factors such as light chain immunoglobulins, histones, actins, MAPK family and mast cells degranulation proteases such as carboxypeptidase and cathepsins, which denote prominent involvement of mast cells; S100A8/9, S100A12, S100A4 and S100P are linked with macrophages and neutrophils involvement and consequential inflammation; (**4**) denotes functional analysis of the proteome study in the form of key Reactome pathways with their P-values and FDRs

6.4 Elucidation of molecular and cellular events in osteophytes and their linkage with OA pathology (Publication 4)

6.4.1 RNA-seq analysis of osteophytes

6.4.1.a Differential transcripts between osteophytes and control specimens

After statistical analysis, 595 differentially expressed genes were found between osteophytes and non-osteophytic controls. Among 595 genes, 322 genes were up-regulated ($\log FC \geq 2$), while 273 were down-regulated ($\log FC \leq -2$) (**Figure 13**). Further, k-means clustering analysis was performed to find out highly up-regulated genes. In this, optimum number of clusters was determined by the elbow method. In case of up-regulated genes, optimal K was 7 and cluster numbers 1, 5 and 7 showed high significance ($P < 0.001$). Similarly, for down-regulated genes K-means clustering was also 7 and hence, the data was divisible into 7 clusters. Among these clusters, only the cluster 1 and 7 showed $P< 0.001$. Highly significant clusters of up-regulated genes were considered for further analysis and discussion to maintain the brevity of the topic. Among 322 up-regulated genes, 87 genes were highly up-regulated ($P < 0.001$). Out of 273 down-regulated genes, 22 genes showed a marked decrease in their expression level ($P < 0.001$). Highly significant up-regulated as well as down-regulated genes are listed in **Appendix Table 1** with their P-value and logFC.

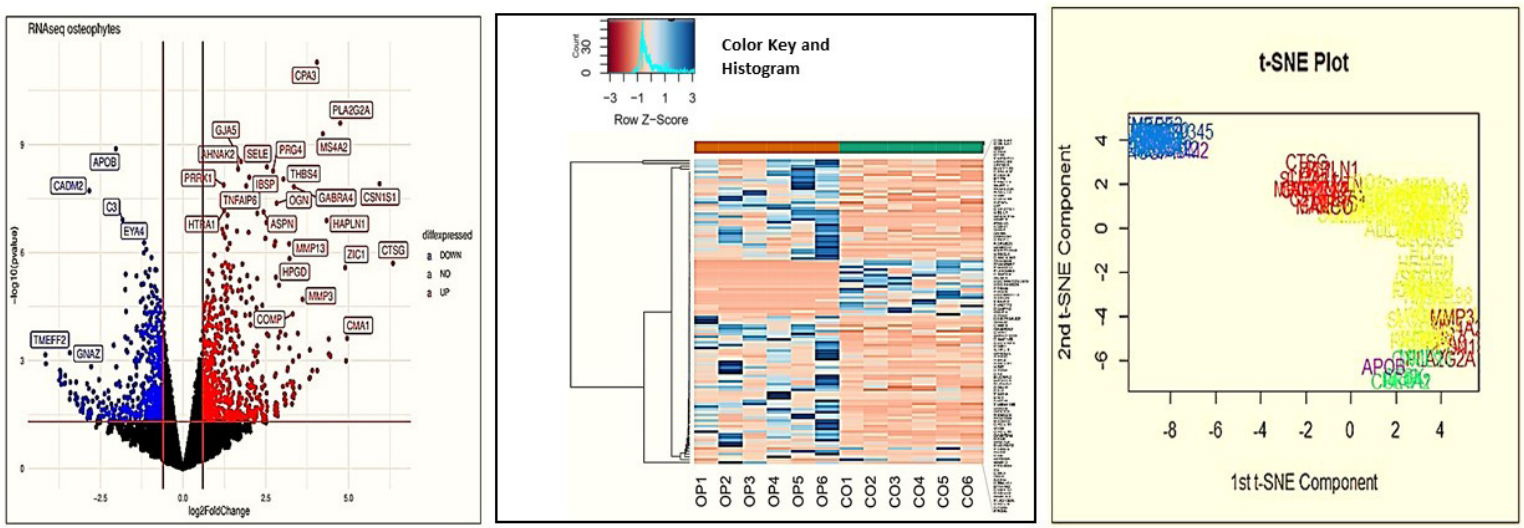


Figure 13: an overview of differentially expressed genes in transcriptome analysis – this analysis was performed using osteophyte samples and non-osteophytic control tissue obtained from six knee OA patients (n = 595); (**a**) volcano plot of the differentially expressed genes. Here, black dots represent insignificant genes, while blue and red dots represent down-regulated and up-regulated genes, respectively. The plot is generated using logFC values of the expressed genes. Carboxypeptidase A3 (CPA3), selectin E (SELE), membrane spanning 4-domains A2/ Fc fragment of IgE receptor 1a (MS4A2/ FcERI), chymase 1 (CMA1), interleukin 1 receptor like 1 (IL1RL1), collagen type 1 alpha 1 chain (COL1A1), COL1A2, MMP-1, MMP-3 and MMP-13 are among the significantly upregulated genes; (**b**) Heat-map of the highly significant genes, which were obtained after K-means cluster analysis; (**c**) t-SNE plot of 'highly differentiated' genes (generated after K-means cluster analysis). t-SNE is a dimensional reduction technique of presenting large gene data sets; in simpler words, it represents a mathematical extract of all the selected data in the form of different variables (generally, 2, 5 or 10 variables are used). We generated a two-dimensional t-SNE plot to verify the cluster analysis. For this we considered logFC, logCPR and P-values of the highly significant genes in the osteophyte specimens.

6.4.1.b Up-regulated genes

Marked increase in the expression of CMA1 (5-fold), CPA3 (4-fold), MS4A2/ FcERI (MS4A2; 4.2-fold) and interleukin 1 receptor-like 1(IL1RL1; 2.5-fold) indicated an active participation of mast cells in osteophytes pathobiology. Additionally, osteophyte specimens were characterized by on-going ECM remodelling as revealed by a marked up-regulation of genes like SELE (2.5-fold), COL1A1 (2.04-fold), COL1A2 (2.01-fold), MMP-1 (3.03-fold), MMP-3 (3.54-fold) and MMP-13 (3.2-fold). Transcriptomics results were validated by qRT-PCR analysis; in this, presence of CPA3, TPSAB1, CMA1, MMP-1, MMP-3 and MMP-13 genes were confirmed and their expression levels are presented in **Figure 14**.

Table 3: Significantly up-regulated mast cell specific genes and their patient-to-patient variation in the transcriptome analysis; logFC showed the difference of the gene expression between OA cases and controls and IfcSE was a standard deviation of the difference

Gene Name	LogFC	IfcSE
CMA1	5.01	1.92
CPA3	4.02	0.59
MS4A2	4.22	0.63
IL1RL1	2.5	0.80

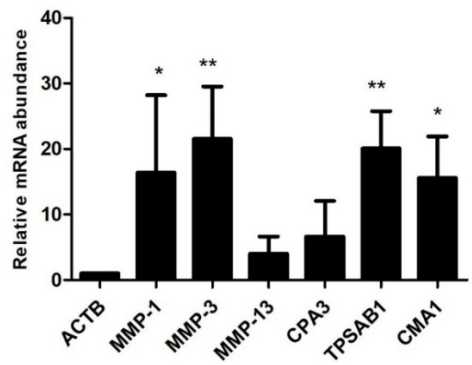


Figure 14: qRT-PCR analysis, as a validation of transcriptome results of the osteophyte samples. mRNA levels of CPA3, CMA1, TPSAB1, MMP-1, MMP-3 and MMP-13 were normalized against ACTB, used as a house keeping gene. All the values are expressed as mean ± SD. TPSAB1 (20.04-fold), CMA1 (15.57-fold), MMP-1 (16.32-fold) and MMP-3 (21.5-fold) showed a significant up-regulation. CPA3 (6.64-fold) and MMP-13 (4.01-fold) also showed up-regulation, which was found insignificant on statistical scale. The validation was limited to confirm the expression of key upregulated genes from the transcriptomics data in the osteophytes. * $P < 0.05$, ** $P < 0.01$, compared to ACTB

6.4.1.c Down-regulated genes

Apolipoprotein B (APOB), cell adhesion molecule 2 (CADM2) and tomoregulin-2 (TMEFF2) showed a marked down-regulation ($P < 0.001$) (**Appendix Table 1**). APOB, which was down-regulated by 2.04-fold, is actively involved in cholesterol transport activity and metabolism of fat-soluble vitamins. On the other hand, CADM2 (down-regulation by 3-fold) is an immunoglobulin and is associated with a range of cellular functions like proliferation, migration, cells aggregation to EMC, cell differentiation and apoptosis. TMEFF2 (down-regulation by 4.15-fold), TNF Receptor Associated Factor 2 (TRAF2) (down-regulation by 3.7-fold) and Protein Inhibitor of Activated STAT 4 (PIAS4) (down-regulation by 3.4-fold) were the other prominently down-regulated genes in osteophyte samples. Among these genes, TRAF2 and PIAS4 encode important cell signalling proteins; moreover, TRAF2 is pivotal for TNF-α mediated activation of MAPK8/ c-Jun N-terminal kinase (JNK) and NF-κB and also anti-apoptotic signal from TNF receptors. PIAS4 is a Signal transducer and activator of transcription 4 (STAT4) inhibitor; STAT4 is a transcription factor for key OA cytokines like interferon-γ and TNF and a signalling factor, MYD88.

6.4.1.d Functional annotation of the differentially expressed gene networks

Functional analysis of the differentially expressed genes in osteophyte samples was limited to up-regulated genes only. A pathway enrichment analysis of the up-regulated genes enabled us to evaluate biological themes in the complex lists of differentially expressed genes. For this, Enrichr software was used; the software has an identified database of 180, 184 annotated gene sets from 102 gene set libraries (Kuleshov et al., 2016). This analysis revealed a significant activation of MMP as a top enriched canonical pathway with a combined score of 17.59 (**Table 4**). The other highly enriched pathways included were – osteoblast signalling (score 16.86), receptor activator of nuclear factor-kappa-B ligand (RANKL/NFκB) signalling pathway (score 16.71), angiotensin-converting enzyme (ACE) inhibitor pathway (score 16.47) and osteoclast signalling pathway (score 15.73) (**Table 4**).

Table 4: Key pathways with their P-values, Z-score and combined score, generated during function analysis of the differentially expressed genes in osteophyte samples

Sr. No	Name of the Pathway (with WikiPathway No.)	P-value	Adjusted P-value	Z-score	Combined score
1	Matrix Metalloproteinases WP129	0.0005743	0.01594	-2.36	17.59
2	Osteoblast Signaling WP322	0.00005487	0.004648	-1.72	16.86
3	RANKL/RANK (Receptor activator of NFKB (ligand)) Signaling Pathway WP2018	0.0002299	0.008505	-1.99	16.71
4	ACE Inhibitor Pathway WP554	0.003794	0.07020	-2.95	16.47
5	Osteoclast Signaling WP12	0.00008374	0.004648	-1.68	15.73
6	Photodynamic therapy-induced NF-kB survival signaling WP3617	0.0009077	0.02015	-1.81	12.69
7	Inflammatory Response Pathway WP453	0.01159	0.1040	-2.65	11.80
8	Oncostatin M Signaling Pathway WP2374	0.005383	0.07468	-1.93	10.06
9	Composition of Lipid Particles WP3601	0.04800	0.2424	-3.27	9.92
10	GABA receptor Signaling WP4159	0.01235	0.1040	-2.18	9.60

Furthermore, involvement of the inflammatory response pathway (WP453) indicated immune-regulation and signalling in osteophyte specimens. Altogether, the enrichment analysis revealed a footprint of bone and cartilage ECM remodelling and inflammatory responses, especially mediated by the canonical RANKL/NFκB signalling pathway.

6.4.2 Transcriptome validation studies (Publication 4)

6.4.2.1 IHC of the osteophytes

In histopathological investigation of the osteophytic tissue, the key observations revealed bone matrix with distinguishable osteoblasts, osteocytes and bone-matrix interspersed with occasional blood vessels arriving from the subchondral bone. The cartilage was noted with distinguished columnar chondrocytes embedded in the cartilage matrix. IHC staining with antibodies anti-TPSAB1 and anti-FcERI confirmed an extensive presence of mast cells in all the osteophyte samples **Figure 15** (**P1**, **P2**, **P3**). Positively-stained cells were profoundly visible in the fibrocartilage and bony tissue of the osteophyte specimens. The stained-cells were grouped in high numbers along with the inner lining of the osteon and also in the space between subchondral cancellous bone trabeculae. Moreover, the cells were observed along the chondro-osseous junction, where the osteophytic cartilage and bone meet.

A frequency of positively-stained cells was determined by the ordinal method as described (Meyerholz and Beck, 2018). In this, anti- TPSAB1 staining incidence range was 1-3% between control and the osteophyte samples except in one osteophyte sample, wherein the incidence range was 40% (**Figure 15 C**, **P1**, **P2**, **P3**- anti-TPSAB1 panel). In this sample, mast cells were gathered in high numbers in the space between subchondral cancellous bone trabeculae (**Figure 15 anti-TPSAB1 panel**). Incidence of anti- FcERI staining in control tissue had a range between 5–30%, while it varied from 10–70% in the osteophyte samples. Statistical analysis of both the antibodies staining showed a significant difference between control and osteophyte samples, $P < 0.01$ (**Figure 15 histograms** – control and osteophytes). In conclusion, IHC study observations further validated a prominent activity of mast cells in osteophyte specimens as revealed in the transcriptome study.

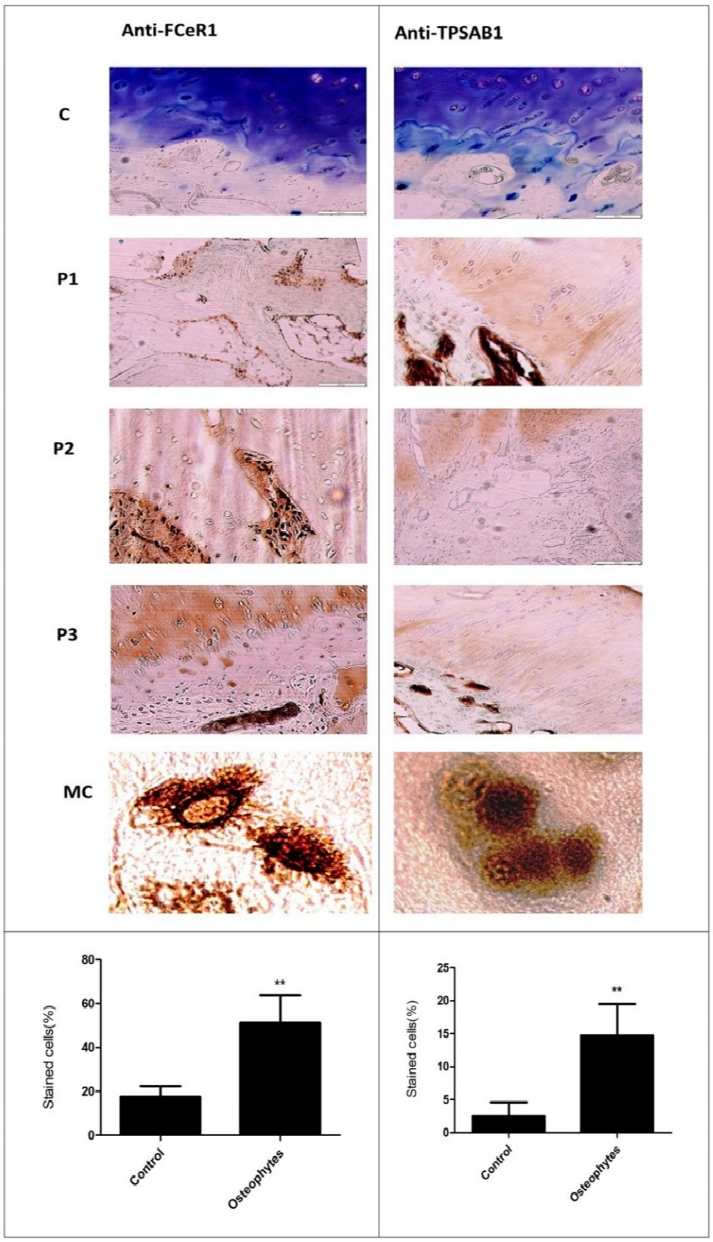


Figure 15: IHC study of osteophyte samples – representative slides of IHC staining with anti- FcERI and anti-TPSAB1 to confirm mast cells in osteophyte sections. P1, P2 and P3 denotes representative areas of osteophytes of study patients. Positively stained mast cells were localized in bone trabeculae, cartilage region, where the columnar chondrocytes can be seen. MC are mast cells at 100X magnification revealing granular features. Bottom panel: comparative histograms between control and osteophytes denoting anti-FcERI and anti-TPSAB1 stained cells (%) respectively; * $P < 0.05$, ** $P < 0.01$ compared to control.

7. DISCUSSION

7.1 Inflammation in synovium-SF axis is a key regulator of OA pathology (Publication 1, 2 and 3)

Although OA is characterized by progressive cartilage damage, studies in last 15 years indicate 'chronic inflammation' as a major pathology responsible for this damage (Goldring and Otero, 2011, Berenbaum, 2013, Mobasheri et al., 2017). With the improved understanding, OA can be defined as a state of skewed balance between pro and anti-inflammatory factors, wherein catabolic processes take over anabolic processes in the synovial joints. The present thesis investigated various molecular and cellular events revolving around the synovium-SF axis. The axis is known for its unique significant biological functions of nurturing, maintaining, and protecting cartilage. It has a major connectivity with systemic circulation and hence, the first systemic insults are received by synovium and relayed through SF. The synovium-SF axis is therefore expected to hold early to advance signs of OA pathology (Kulkarni et al., 2021).

Synovium gene expression study was a purposive approach to study a variation pattern of inflammatory mediators at different stages during natural OA progression. A definite expression trend was noticed in IL-1β, IL-15, PGE2 and NGF, key inflammatory markers of OA pathology. The highest expressions of IL-1β and IL-15 in KL grade-I synovial biopsies indicated that these inflammatory regulators are present in excess since early stage of the disease that provides a biochemical trigger even before the appearance of definite clinical signs of OA. Peak of PGE2 was found in KL grade-II samples, immediately in the next stage of high IL-1β, which is the main inducer of PGE2. NGF showed comparable expressions in synovial biopsies of KL grades-I, III and IV with the lowest expression in KL grade-II biopsies. A grade-wise comparison for IL-1β and IL-15 expression showed a marked significance in KL grade-I synovium as compared to other grades; however, PGE2 and NGF did not reveal any significant expression change, possibly due to a small number of samples. Gene expression study of synovium biopsies thus underlined a persistent nature of inflammation present in OA joints, unlike a common claim that synovitis is prevalent at early and advanced stages. Moreover, these results indicated that each stage of OA can be marked by a presence of a particular inflammatory factor. Individual role of the selected pro-inflammatory factors has been extensively studied in OA and their increased levels has been recorded in OA affected clinical samples (Hardy et al., 2002, Scanzello et al., 2009, Walsh et al., 2010, Kokebie et al., 2011). In brief, increased levels of IL-β, IL-15 and PGE2 are associated with impaired bone and cartilage remodeling. Particularly, IL-1β and IL-15 are known to cause damaging effects on articular cartilage by up-regulating MMPs (Scanzello et al., 2009, Wojdasiewicz et al., 2014). PGE2 and NGF are pro-angiogenic factors and their high levels contribute to synovitis

and synovial hyperplasia; NGF also revealed a strong connection with transmission of pain in OA (Sellam and Berenbaum, 2010, Walsh et al., 2010, Wojdasiewicz et al., 2014,). Based on the study outcomes as presented in Kulkarni et al., 2016 and Ingale et al., 2021, the authors claim a possibility that a combined effect of elevated IL-β, IL-15 and PGE2 in early stages of OA provides a necessary impetus for accelerated cartilage loss in later stages of the disease as was evident by relatively higher expression of MMPs in advanced staged SF samples (KL grade-II and III) and synovial biopsies (KL grade-III and IV).

SF is the other counterpart of this axis and accumulation of pro-inflammatory factors in excess amounts has been reported in OA SF samples by our group and others (Wojdasiewicz et al., 2014). In our Publication 1 and other previously published studies, pro-inflammatory mediators like IL-1β, TNF-α, NO and NN estimated in SF samples of different degree of OA showed a peculiar trend, wherein the highest level of these markers was found in early and moderate OA cases (KL grade-I, II and III). These levels were seen declined in most of the advanced OA samples (grade IV), possibly as a result of total worn out of cartilage (Kulkarni et al., 2014, Koppikar et al., 2015, Koppikar, 2017, Ingale et al., 2021).

Inflammatory factors in OA SF samples may act as trigger for cellular and tissue inflammation in the disease pathology (Koppikar et al., 2015). These proteins however are needed to be tested for their potential to induce OA relevant inflammation in the joint tissues individually and collectively to comprehend the underlining inflammatory mechanism. SF challenge test of this thesis was designed to determine SF's capacity to provide inflammatory microenvironment of the joint in terms of estimation of pro-inflammatory mediators like NO, MMP-1 MMP-13 and VEGF-1.

Hypothesis of SF challenge test was based on our previous learning that OA SF holds inflammatory milieu of various cytokines and chemokines, which can be used to study the disease specific responses on cell lines (Koppikar et al., 2015). In the present thesis, this test was performed on two different human monocyte cell lines – THP1 and U937, wherein, the cells were treated with SFs of different KL grades for 48 hours. As the cells were subjected to OA similar inflammatory micro-environment, their response was expected to mimic as *in vivo* conditions of the joint. Inflammatory response of these SF treated cells was measured by released NO levels and mRNA abundance of pro-inflammatory markers like MMP-1, MMP-13 and VEGF-1 (**Figure 5** and **6**). Excessive NO is released by the stressed cells as an indicator of inflammation; further, this unmitigated NO stimulates MMP production and inhibits synthesis of collagen and proteoglycans in OA joints (Goldring and Otero, 2011). In the SF challenge test, THP1 as well as U937 cells after 48h of SF induction showed a significant NO release as compared to untreated cells, was a clear indication of cellular inflammation (**Figure 5A** and **6A**). Inflammation induction was reconfirmed by evaluation of gene expressions of pro-inflammatory markers like MMP-1, MMP-13 and VEGF-1. Interestingly, both the cell lines showed upregulation of

MMP-1 and MMP-13 in a similar manner; this means that early to moderate grade OA SFs (KL grade-II and III) caused the highest up-regulation of MMP-1. For THP1 cells, maximum up-regulation of MMP-13 was found after SF treatment of KL grade-IV, while its expression remained comparable in all KL grades in case of U937 and significantly higher than control. MMP-1 and MMP-13 serve as surrogate markers of inflammation. In OA joints, responding to local and systemic insults, synoviocytes and chondrocytes release a number of cytokines including IL-1β and TNF-α, which are the major stimulators of catabolic factors like MMP-1 and MMP-13 (Goldring and Otero, 2011). Moreover, DAMPs released in SF act as a legend for TLR-2 and 4 to generate an inflammatory response, which further leads to increased expression of MMP-1 and MMP-13. Thus, significant up-regulation of these two genes after the SF treatment was an indicator for successful induction of inflammation (Goldring and Otero, 2011, Wojdasiewicz et al., 2014). Additionally, SF induced THP1 and U937 cells showed a significant up-regulation of VEGF-1, a pro-angiogenic factor. Interestingly, Hoff et al., 2013 showed that OA SF is more potent inducer of VEGF in primary chondrocytes as compared to RA SF, indicating that pro-inflammatory mediators in OA SF could induce inflammatory changes in synoviocytes as well as in chondrocytes too.

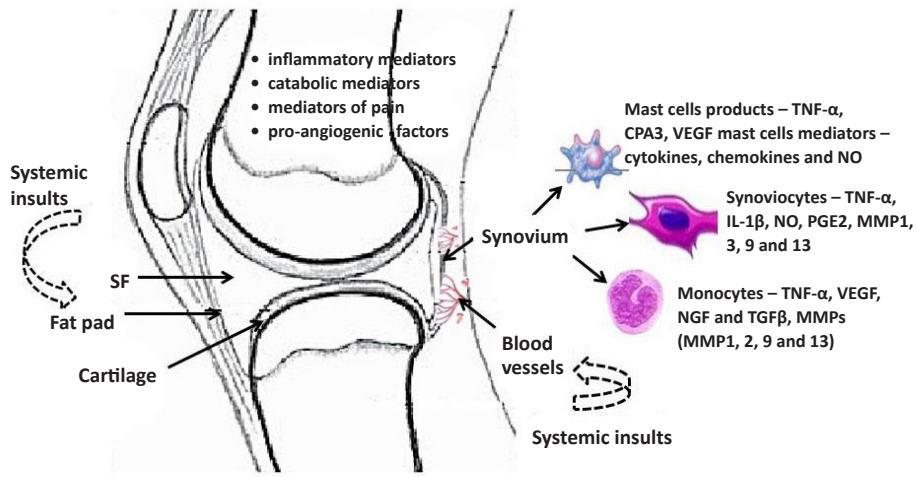


Figure 16: Synovium-SF axis in OA pathology – being connected to systemic circulations, synovium is able to mediate systemic insults to joint; synoviocytes and immune cells including monocytes and mast cells, which are major infiltrates of OA synovium contributes to the key cytokines like IL-1β, IL-15 and other pro-inflammatory mediators like NO and VEGF that induce inflammation and angiogenesis and stimulate excess production of MMPs (like, MMP-1 and MMP-13) ultimately leading to cartilage loss; additionally, mast cells degranulation proteases like CPA3, are also known to induce sterile inflammatory responses; besides a communication channel of the joint, SF also able induce cellular inflammation through its disease specific cocktail of cytokines, chemokines and inflammatory factors

7.2 OA SF act as a niche in inducing immune cell differentiation and providing them an 'effector status' (Publication 4 and 5)

A concept of 'niche' represents a developmental dynamic between immune cells and their adaptation to tissue-derived signals. Immune cells are actively engaged with each other and to their tissue-specific microenvironment or 'niche' to be functionally imprinted by tissue-specific cues. Thus, niches nurture these cells with a physical scaffold for anchoring and survival factors, as elaborately explained in case of macrophages by Guilliams and Scott, 2017 and Guilliams et al., 2020. In the state of inflammation like OA, circulatory immune cell progenitors enter into the affected joints and infiltrate OA synovium to occupy the vacant site due to a partial depletion of tissue resident macrophages and other immune cells. The recruited cells further adopt a tissue-specific identity and take up a role similar to resident immune cells (Laar et al., 2016), wherein a niche is needed for functional adaptation of the cells. Although the exact nature of niche still unclear, a common understanding in this regard is that, this functional adaptation and macrophages polarization is highly regulated by cues in the tissue microenvironment including cytokines, growth factors, and microorganism-associated molecular patterns. These factors in the tissue microenvironment are thought to educate the recruited immune cells about their phenotype and functions on the physiological or pathological context (Italiani and Boraschi, 2014).

The findings of SF challenge test experiments of this thesis and the other studies published by the author showed that the protein make-up in OA SF can induce cellular inflammation (Koppikar et al., 2015, Ingale et al., 2021, Kulkarni et al., 2021); therefore, it was logical to further investigate if the induced inflammation can modulate immune cell differentiation process, which are the major precursors of joint inflammation, creating a vicious circle in OA pathology. In other words, *in vitro* cell differentiation assays were designed to determine 'functional involvement' of SF in OA pathogenesis. For this, different cell types (U937, THP1 and HSCs) were treated as immune cell precursors and incubated with OA SFs of different disease severity for 48h to modulate the cell differentiation process. The assay design was based on the learning that, *in vitro*, macrophages polarization can be achieved by exposing monocytes to different inducing agents like IFN-γ, lipopolysaccharide (LPS), PMA, IL-1β and TNF-α to develop pro-inflammatory macrophages (Schwende et al., 1996, García et al., 1999, Liu et al., 2005, Daigneault et al., 2010, Sintiprungrat et al., 2010, Hsueh et al., 2020). To our knowledge this is the first report, wherein OA SF with its pathobiological protein milieu of was used as an immune cell differentiation inducing agent.

The outcomes of the *in vitro* assays showed that SF treatment to U937, THP1 and HSCs induced a differentiation and caused a phenotype change in them. U937 cells after 48h of SF induction were found differentiated into

CD14+, CD86+, CD163+ phenotypes. In the selected antibody panel for U937, CD163 denote M2 macrophages, while CD86 represent M1 macrophages; CD14 is a pan macrophage marker. In the *in vitro* assay on THP1 and HSCs, the cells were induced for 9 days; here, THP1 cells showed a clear differentiation into HLA-DR+ and CD206+ phenotypes and HSCs also showed a marginal differentiation into FcERI+ phenotype. In the antibody panel for THP1 cells, HLA-DR and CD206 indicate M1 and M2 phenotype, respectively. A grade-wise SF induction pattern showed a significant increase in M2 phenotype after the SF induction with early OA samples (KL grade-II) as was evident by an elevation in the percentage of CD163+ and CD206+ phenotype (**Figure 8D**). This differentiation pattern was consistent for SF induced U937 and THP1 (**Figure 8D** and **Figure 9a1**). The outcome has many aspects; as said earlier, *in vitro* human macrophages polarization is reported to be influenced by sequential changes in tissue microenvironment. Therefore, a significant increase in M2 phenotype (CD163+ and CD206+) following the treatment with SF of KL grade-II was possibly a natural combating response to high degree of pro-inflammatory milieu in the KL grade-II SF samples that was seen declined in the later stages. The SF induction with KL grade-III and IV showed a decline in M2 phenotype and a simultaneous significant increase in CD86+ (**Figure 8C**). In THP1 cells, SF treatment with KL grade-II and III showed a comparable number in HLA-DR+ cells. As reported in our previous study, SF samples of KL grade-II and III were able to generate the highest cellular inflammation and hence, can be considered for having higher levels pro-inflammatory factors (Koppikar et al., 2015).

Despite of higher pro-inflammatory milieu, a decline in CD163+ phenotype after KL grade-III SF induction can be designated to insufficiency of the combating efforts to neutralize the increasing inflammatory microenvironment and a possible switching of polarized macrophages from one phenotype to other as suggested (Stout et al., 2005, Mylonas et al., 2009, Egawa et al., 2013, Italiani et al., 2014). Accounting these studies and SF as the inducing agent, which held a varied protein milieu, reflecting a stage of OA progression, there is a scope for the inference that similar switching from M2 phenotype to M1 phenotype had taken place. In fact, similar kind of the phenotype switching is reported in the chronic metabolic disorders like, obesity, type-2 diabetes and atherosclerosis (Bastard et al., 2006, Martín-Fuentes et al., 2007). Of note, obesity and type-2 diabetes are well known confounding factors of OA (Sellam and Berenbaum, 2013). Therefore, the possibility of switching the macrophage phenotype holds strong especially *in vivo* conditions of OA joints, wherein persistent low grade inflammatory microenvironment is maintained. In this regard, the authors acknowledge that the present study was pilot in nature and they did not show actual switching of the macrophage phenotypes. The SF induction with KL grade-IV showed a comparable stained cell population to the induction with KL grade-III as was found in U937 experiment, which indicates a phenotype shift in transit from KL grade-III to IV. This also points out a comparable existing pro-inflammatory protein make-up of the SF samples of

KL grade-III and IV used for the induction (**Figure 11** and **Appendix Table 3**). Functionally active status of the differentiated cell confirmed by the cytokine estimation also coordinated with their phenotypic shift (**Figure 8** and **Figure 10**). Unlike the assay on U937 cells, the induction with SF of KL grade-IV in THP1 showed some decline in the percentage of M1+ cells (HLA-DR+), which can be attributed to a possible total cartilage warn-out effect in the terminal OA SF samples, which ultimately cause a reduction in the associated inflammation. The two distinct categories of biomarkers found in KL grade-IV SF samples, the pattern of inflammation induction of these samples and their correlation with cartilage-loss are elaborately discussed in our previous publications (Kulkarni et al., 2016, Koppikar et al., 2015, Ingale et al., 2021). The collective evidence of *in vitro* cell differentiation assays on U937, THP1 and HSCs indicated a decisive role of OA SF in the differentiation of polarized cells into effector cells such as mast cells and macrophages. Of note, the cell differentiation assays on THP1 and HSCs were performed as a part of osteophytes study, wherein based on the IHC outcomes the authors propose SF mediated mast cells invasion into osteophytes (**Figure 9**). The IHC study of osteophytes revealed extensive population of anti-TPSAB1 and anti-FcERI-stained cells in cartilaginous region, which is directly exposed to SF in the joint cavity (**Figure 15**).

A study by Kraus et al., 2016 reported a positive correlation between activated macrophages and OA severity, providing the first direct *in vivo* evidence for microphages involvement in OA pathology. Further, on a similar theme, the status of the activated microphages was determined in terms of M1 and M2 subtypes in OA SF samples by Liu et al., 2018. This study further showed that a ratio of M1/M2 macrophages was significantly higher in knee OA patients as compared to healthy controls and further correlated with the disease severity. On similar notes, M1/M2 ratio calculation in the newly differentiated cells in this study was another functional validation of SF potential to induce immune cells differentiation. M1/M2 ratio in the newly differentiated cells of *in vitro* assay on U937 cells revealed similar findings; the ratio was significantly higher in the cells induced with SFs of KL grade-III and IV as compared to untreated cells (**Figure 17**). To calculate this ratio, the average percentage of all M1+ cells (CD86+) was divided by the average percentage of M2+ cells (CD163+) in each KL grade. We did not consider the results of THP1 assay for the ratio calculation because of different SF induction time point. M1 dominated polarization of macrophages has anti-chondrogenic effects and increased production of proteolytic enzymes including MMP-13 and ADAMTS5 (Fahy et al., 2014, Utomo et al., 2016). Accounting these observations, it can be said that significantly higher M1/M2 ratio as found in the newly differentiated cells in U937 *in vitro* assay after induction of KL grade-III and IV SFs correlates well with the KL score system and can be seen as a potential predictor of OA severity. Nonetheless, these ratio observations also underlined SF's role in driving M1 dominated macrophages polarization.

Finally, the percentage of differentiated immune cells after SF induction should be interpreted on the basis of macrophage and mast cell proportion as evaluated in the immunophenotying experiment (**Figure 7**). The highest percentage of macrophages (estimated as CD14+ cells) was found in KL grade-II fluid samples and were comparable to the other reports, wherein the immune-profiling of macrophages in OA SFs was performed using a combination of surface markers like, CD14, CD86 and CD163 or CD14 and CD16 marker (Kriegova et al., 2018, Gomez-Aristizabal et al., 2019). In our study, we used CD86 and CD163 markers as representative of M1 and M2 type of macrophages, respectively; the percentage of both the types in our SF samples was found negligible. Also, comparatively lower number of differentiated FcERI+ cells after SF induction in HSCs should be interpreted on the basis of mast cells proportion in OA SFs as reported by the other authors **(**Malone et al., 1986, Dean et al., 1993, Kriegova et al., 2018). We restricted our SF cells immunophenotyping study to macrophages and its subtype estimation. Percentage of mast cells in OA SF samples was reported as 0.3% of the total cell population (Kriegova et al., 2018). SF samples from minimal OA and established OA revealed the cell percentage as 1.05 ±0.97 % and 0.84 ± 0.82 % of the leukocytes in all fluids, respectively (Dean et al., 1993). Similar number of mast cells were reported OA SF samples by Malone et al, 1986.

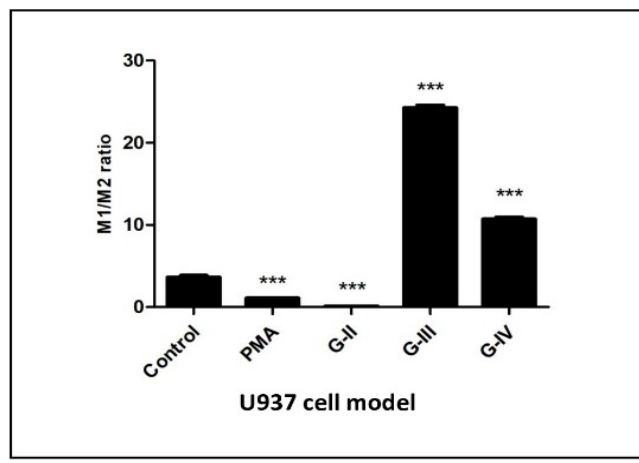


Figure 17: a grade-wise estimation of M1/M2 ratio in the newly differentiated cells after SF induction for 48h in U937; the differentiation status of these cells was assessed using CD14, CD86 and CD163; M1/M2 ratio was estimated by dividing the average value of M1 marker (CD86+) with the average value of M2 marker (CD163+) in each KL grade. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, as compared to control

7.3 Elucidation of immune cells regulatory factors in OA SFs (Publication 4 and 5)

Proteome analysis revealed that OA SF encloses a range of up-regulated proteins that are associated with immune cell regulation. Differentially expressed proteins like Ig light chains and free histones in all grades of SFs are mast cell regulatory proteins (**Appendix Table 2**). Ig light chain proteins (kappa and lambda variable) can bind to FcERI and FcgRI receptors on mast cells and trigger a hypersensitive response (Redegeld et al., 2002). Free histones activate immune cells by inducing an inflammation via TLR4 and TLR2 receptors (Xu et al., 2009, Xu et al., 2011) (**Figure 12**). Thus, free histones accumulated in SFs after cell death are classical nuclear presentation of DAMPs and ultimately elevate inflammation by stimulating various immune cells including mast cells. Interestingly, our thinking is reinforced by Tasaka et al., 1990, wherein exposure of rat peritoneal mast cells to a mixture of histones resulted in instant degranulation and the release of histamine, indicating that mast cells readily respond to free histones. Transcriptomics analysis of the osteophytes showed a significant elevation in MAS related G protein-coupled receptor-X2 (MRGPRX2) (4.5-fold), while its receptor neurotensin was detected in high levels (37.18-fold) in the proteome analysis. When seen collectively, this is an indicator of an existing IgE independent pathway of mast cell activation in osteophyte samples. This is because, MRGPRX2 binds to cationic ligands, neuropeptides and opioids and, therefore, represents IgE independent pathway of activation. Both the pathways (IgE dependent and independent pathway) trigger distinct patterns of secretion of mast cell mediators (Meixiong et al., 2019). The outcomes of IHC osteophytes and *in vitro* cell differentiation assays on HSCs provided a clear indication that besides subchondral bone, an alternate route for mast cells invasion into osteophytes exists and deeper confirmation studies along this line should take place (**Figure 18**).

Significantly up-regulated proteins that have major effect on functioning of macrophages are enlisted in **Appendix Table 3**. In the proteome analysis, microphage migration inhibitory factor (MIF) was noticed progressively down-regulated across the KL grades. MIF is an immunoregulatory cytokine that arrest random immune cell movement, promotes cell recruitment and is also responsible for M1/M2 polarization. In a xenograft model of glioblastoma down regulation of MIF led to increased macrophages at the edge of the tumor (Castro et al., 2017). In the current context, MIF may be responsible for maintenance of macrophage population in the joint tissues. A significant increase in macrophage-capping protein (MCP) (40-fold in the present study) was recently showed to influence a migration behavior of macrophages by modulating actin dynamics through capping the growing end of actin filaments (Prescher et al., 2021). Grancalcin is a calcium binding protein found increased by 42-fold in KL grade-III and IV SF samples; this protein is involved in controlling calcium influx and modulates secondary signaling in the effector cell. Many RAS-related proteins were found significantly upregulated especially in KL grade-III

and IV SF samples; these proteins are involved in various functions of macrophages such as ER trafficking, and phagocytosis. Members of RAB family proteins are low molecular mass monomeric GTPases localized on the cytoplasmic side of membranes, these proteins are involved in various functions of macrophages such as ER trafficking, secretory pathways and are essential for vesicle transport (Karimi et al., 2018). Osteopontin (showed 35.95-fold expression in grade 4), is a multifunctional protein involved in various signaling pathways. A recent publication by Liu et al 2022, propose osteopontin to affects macrophage polarization promoting endocytic activity but not inflammation. As a recently described protein family, tumor necrosis factor alpha induced protein-8 (TNFAIP8) plays role in maintaining immune homeostasis and inflammatory response. Silencing TNFAIP8 protein using siRNA dramatically decreased IL-1β secretion in RAW264.7 macrophages (Zang et al., 2018).

Up-regulation of S100 proteins such as S100A8, S100A9, S100A12 and S100A4 in all grades of SF samples, was another classical feature of OA. These proteins were reported in excess in early phase of OA (Zreiqat et al., 2010); their plasma levels were found high at baseline in human OA participants. Increased expression of S100A8 and S100A9 can be linked to excess oxidative stress and cytokine production in OA (Hsu et al., 2009). An antigen-induced arthritis model confirmed their contribution in cartilage degradation and development of inflammatory arthritis (Van Lent et al., 2008), whereas $S100A9^{-/-}$ mice-induced OA model showed a dramatic decrease in osteophyte size (Schelbergen et al., 2016). Extracellular S100 proteins released from the cells after cell damage/stress or activation of phagocytes (such as neutrophils and macrophages), serve as DAMPs and activate inflammatory response by binding to TLRs and receptor of advanced glycation end products (RAGE) (Kligman et al., 1988, Winningham-Major et al., 1989, Vogl et al., 2007, De Haan et al., 2013). S100A12, S100A8/A9 binding to TLR4 and RAGE activates NF-κB, inducing the production of pro-inflammatory cytokines leading to the migration of neutrophils, monocytes, and macrophages (Vogl et al., 2007, Gebhardt et al., 2008, Ghavami et al., 2008, Turovskaya et al., 2008, Sorci et al., 2013, Gross et al., 2014). Additionally, MAPK-mediated signaling is also induced by S100 proteins such as S100P (Maletzki et al., 2012, Arumugam et al., 2013).

S100A8 and S100A9 are predominately expressed in monocytes, neutrophils and dendritic cells (Edgeworth et al.,1991, Averill et al., 2011); upon activation, the proteins were also seen expressed in fibroblasts (Rahimi et al., 2005). Expression of S100A12 is reported in the cell types such as, neutrophils, monocytes, and early macrophages (Ravasi et al., 2004, Hsu et al., 2009). A role of these proteins in neutrophils as well as macrophages activation is well recognized. The alarmins, S100A8/A9 not only facilitate monocyte and neutrophil transmigration (Eue et al., 2000, Nisapakultorn et al., 2001) but also induce pro-inflammatory cytokine production in monocytes and macrophages through NF-κB and p38 MAPK pathways (Sunahori et al., 2006). These proteins were showed to induce apoptosis and autophagy in macrophages, endothelial cells,

and tumor cells, wherein involvement of ROS molecules like BCL2 Interacting Protein 3 (BNIP3) is critical (Ghavami et al., 2010). S100A4 also promote cell migration by interacting with cytoskeletal proteins; deletion of this protein leads to the deficiency of macrophage migration and chemotactic reactions (Garrett et al., 2006, Boye et al., 2010, Malashkevich et al., 2010). S100A12 is reported to induce production IL-6 and IL-8 in both a dose-dependent and time-dependent manner, which is critical to regulate the recruitment of monocytes and TNF-α release (Yang et al., 2007). In summary, the proteome analysis of SF samples revealed a variety of different proteins that are present in excess and capable to induce immune cell differentiation as was evident in the outcomes of the cell differentiation assay.

7.4 Elucidation of molecular and cellular mechanisms in osteophytes and their linkage to OA pathology (Publication 4)

Transcriptome analysis of the osteophyte samples showed a significant up-regulation of the transcripts like cathepsins, COL1A1, COL1A2, SELE and MMPs. All these genes reflected an ongoing bone and cartilage remodelling in the osteophyte samples. Among the cathepsins, cathepsin K (2.24-fold) is known for its active involvement in bone resorption (Zhao et al., 2009) and was detected in the zones of active bone remodelling in osteophytes along with the other cathepsins (Lang et al., 2000). On the other hand, cathepsin G (6.38-fold) is one of the proteases of mast cells granules, that stimulates MMPs (Krystel-Whittemore et al., 2016) and is also involved in the activation of osteoclasts and osteolytic lesions by mediating the release of a soluble form of RANKL from the bound RANKL present on the surface of osteoblasts (Wilson et al., 2009). **Table 4** demonstrates key signalling pathways, detected in the functional analysis of expressed gene network in the osteophyte samples; the RANKL signalling pathway was detected as one of the activated pathways in the specimens. Moreover, significant up-regulation of the bone matrix proteins like COL1A1 and COL1A2 indicated the presence of fully mature osteoblasts in the osteophytes (Komari, 2010). Interestingly, growth factors like TGF-β and BMPs, which are attributed to trigger and maintain the growth of osteophytes (Blom et al., 2004, Gelse et al., 2012), were not found up-regulated in our transcriptomics. This was possibly because the collected osteophytes were fully grown (as they were obtained from the OA patients, undergoing knee replacement surgery) and likely to be in the senescence and hence, were unlikely to reflect any developmental changes.

MMP-1 (3.02-fold), MMP-3 (3.53-fold) and MMP-13 (3.19-fold) were among the prominently up-regulated genes. Expression of MMP-1 and MMP-3 was reported in different areas of osteophytic tissue (Bord et al., 1997), whereas a presence of MMP-13 and MMP-9 was showed in osteophytic cartilage (Gelse

et al., 2012). MMP-13 expression was also reported in osteoblasts, isolated from osteophytes of OA joints and further predicted to contribute to the worsening of OA pathology (Sakao et al., 2009). Thus, our transcriptomics outcomes for MMPs were in line to the other similar studies. Interestingly, ECM remodelling in osteophytes has a mechanistic similarity with endochondral ossification in growth plate formation. In ECM remodelling of osteophytes, proliferating chondrocytes express collagen type-II, whereas the hypertrophic chondrocytes express collagen type-X and MMP-13. On the other hand, in the process of endochondral ossification, osteoblasts express MMP-13 and collagen type-I, whereas osteoclasts express MMP-9. Expression of MMP-1, MMP-3 and MMP-13 was validated by qRT-PCR (**Figure 14**); this validation was limited to confirm the presence of these up-regulated genes. Of note, we were unable to compare the expression levels of the selected up-regulated genes between osteophytes and control tissue due to limited RNA quantity of the control tissues.

7.4.1 Transcriptomics and IHC analysis of osteophytes revealed an active involvement of mast cells (Publication 4)

All the osteophyte samples showed a significant up-regulation of CMA1 (5-fold), CPA3 (4.02-fold), MS4A2/FcERI (4.22-fold) and IL1RL1 (2.5-fold). **Table 3** presents patient-to-patient variation of these genes. Prominent expression of these genes indicated an active involvement of mast cells in the molecular events associated with osteophytes. CMA1 and CPA3 are mast cell degranulation products and were found involved in the degradation of endogenous proteins (Wernersson and Pejler, 2014). MS4A2/ FcERI is a high-affinity IgE receptor and stimulates mast cell degranulation (www.uniprot.com). IL1RL1 is a receptor for IL-33 cytokine and belongs to the TLR superfamily. Activated mast cells synthesize IL-33 *de novo*; this cytokine is known as a mediator of sterile inflammation and found in excess in many allergic diseases as well as in RA, psoriasis and atherosclerosis. Importantly, IL-33 binding to its receptor induces mast cell differentiation, survival and chemotaxis and further activates them to produce various cytokines (Varvara et al., 2018, Olivera et al., 2018).

Marked up-regulation of phospholipase A2 group IIA (PL2G2A) (4.64-fold) and MMP-13 (3.19-fold) in osteophyte specimens also reflect a prominent mast cells activity in the osteophytes and their inflammatory repercussions in OA pathogenesis. PLA2G2A is known as ‘inflammatory secretory phospholipase A20 (sPLA2) (Reddy et al., 1997) and encodes secreted phospholipase A2, which hydrolyze the Sn2 position of phospholipid molecules and usually release diacyl-glycerol molecules and unsaturated fatty acids such as arachidonic acid that ultimately promotes biosynthesis of inflammatory prostaglandins (Reddy et al., 1997). The gene is also reported as one of the components of mast cells granules and its redistribution from granules of resting mast cells to fusion sites and plasma membrane facilitates exocytosis in mast cells of bone marrow origin

(Bingham et al., 1999, Enomoto et al., 2000). Murakami et al., 1993 showed that in bone marrow-derived mast cells exogenous PLA2G2A can stimulate mast cell degranulation and thus indicates mature status of mast cells. An involvement of PL2G2A was reported in inflammatory pathologies such as RA and psoriasis (Reddy et al., 1997). A positive correlation between proteolytic activity of MMP-13 and number of tryptase-positive mast cells in periapical lesions of inflammatory periodontitis indicate involvement of mast cells in active bone remodelling and further suggest a similar role of these cells in osteophyte pathobiology (Andrade et al., 2017). Mast cell-specific proteases like tryptase and chymase are also responsible for bone and cartilage remodelling and release cartilage and cellular degradation products, which are similar to DAMPs. These cartilage and cellular breakdown products interact with TLRs and generate a sterile inflammatory response (Johnson et al., 2003). Osteophytes therefore can serve as a source of breakdown products further promoting inflammatory consequences and worsening of OA pathology. Thus, the collective evidences of the transcriptome data present mast cells as effector cells in the osteophyte pathobiology, contributing to the on-going bone remodelling process. Additionally, mast cell degranulation proteases act as stimulants of inflammation in OA joints.

Accounting the findings of transcriptome analysis, which showed a link between osteophytes and mast cells, the author further investigated for the possible origin of mast cells in osteophytes. At first, subchondral bone appears to be a logical route for mast cells invasion into osteophytes, given that MCPs are known to originate in bone marrow by haematopoiesis (Galli et al., 2020) and osteophytes also have minuscule bone marrow depending on its size and maturity. However, there is no research literature available endorsing this route; furthermore, this route does not explain a process of MCP maturation into effector mast cells. Indeed, our IHC study observation wherein, anti-TPSAB1-stained cells and anti-FcERI-stained cell were seen in large number in the space between subchondral cancellous bone trabeculae in the osteophyte samples, supports the purview that subchondral bone marrow could be a source of mast cells in osteophytes. This IHC study also showed an extensive accumulation of the antibodies-stained cells in the cartilaginous region as compared to the subchondral bone. This observation is suggestive of the mast cells invasion into osteophytes through cartilaginous region and further populate in the tide mark of the cartilage-subchondral bone junction. Anti-TPSAB1-staining was also seen in various zones of cartilage and subchondral bone. This extracellular tryptase in these tissues can be interpreted as a sign of degranulation of mast cells. The authors additionally present the results of *in vitro* assays on HSCs (as explained in subsection 7.2 and 7.3 of the discussion) to support the possibility of the mast cells invasion into osteophytes through cartilaginous region is mediated by SF, which provides a necessary biochemical environment for MCPs maturation into the functional cells.

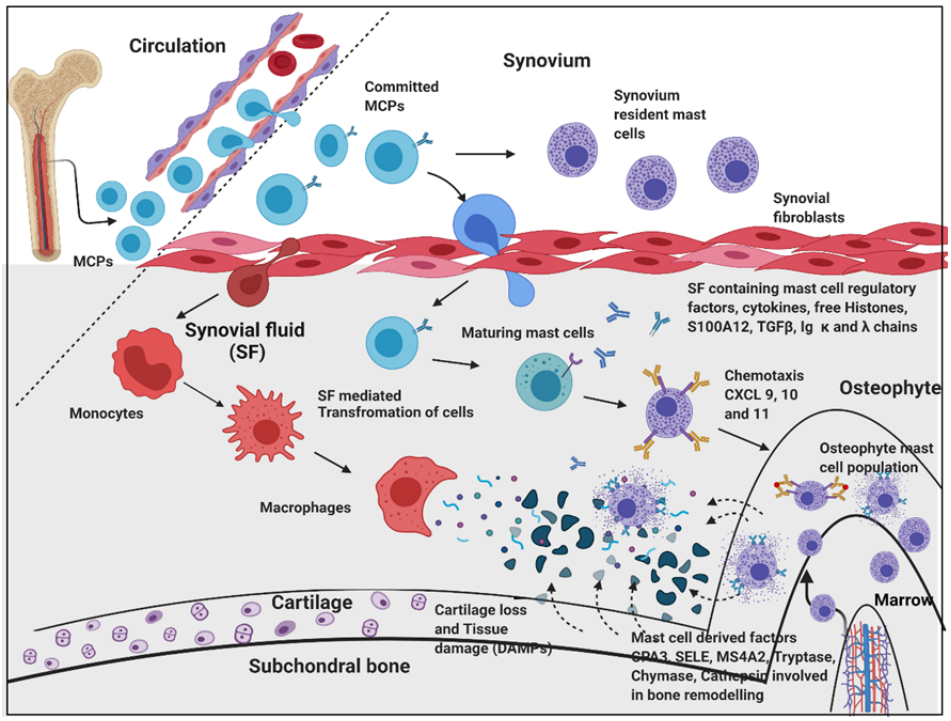


Figure 18: A schematic presentation of a maturation journey of circulatory MCPs, a role of SF in the MCP maturation process and active involvement of mast cells in the osteophytes and its linkage with OA pathology – circulatory MCPs are the major source of mast cells in osteophytes. OA SF, which is composed of cytokines and mast cell regulatory factors like histones, S100A12 and IgG kappa and lambda chain, play an important role in the transformation of MCPs and monocytes into effector cells like mast cells and macrophages, respectively. Responding to the chemotactic signals (CXCL 9, 10 and 11) mast cells migrate and populate osteophyte. Mast cell degranulation factors like CPA3, tryptase, chymase, cathepsins, and SELE are involved in elevated ECM remodelling of osteophyte. These DAMPs accumulate in SF, which act as inducers for mast cell via TLR-2/4 pathway. Increasing action of active mast cells and macrophages contribute for synovitis, tissue damage, and cartilage-loss. This schematic presentation is created with BioRender.com (accessed date – 18 August 2021).

8. CONCLUSIONS

1. Synovium gene expression study revealed a persistent nature of inflammation present in OA joints and further highlighted a differential expression trend of inflammatory markers at each stage of OA progression. For example, the key OA cytokines (IL-1β and IL-15) were significantly expressed in the synovium biopsies of KL grade-I. The presence of these inflammatory regulators in excess at the beginning of early stage (doubtful OA) is likely to provide a biochemical trigger for OA development, even before the appearance of definite clinical signs. In other words, a combined effect of elevated IL-β and IL-15 in early stages of OA provide a necessary impetus for accelerated cartilage loss at later stages of the disease as was showed by the author in her previously published study (Kulkarni et al., 2016). On the other hand, SF analysis for the estimation of key pro-inflammatory factors revealed a significantly higher levels in KL grade-II and III samples, which further correlated well with the outcomes of SF challenge test (Publication 1 and 2).
2. The findings of SF challenge test unanimously indicated that pro-inflammatory factors in OA SF were able to induce inflammation in THP1 and U937 cells. The highest level of NO was estimated after the induction with early and moderate grade (KL grade-I/II/ III) SF samples. These results agree with pro-inflammatory mediator levels in SF samples as well as gene expression of pro-inflammatory markers including MMP-1, MMP-13 and VEGF-1, evaluated in the SF induced THP1 and U937 cells. Importantly, the outcomes of synovium gene expression study and SF challenge test (when seen together) present 'KL grade-II and III' of OA as prominent inflammatory stages (Publication 1, 2 and 3).
3. OA SF induced U937, THP1 and HSCs at different time points showed a clear differentiation into effector macrophages and mast cells, respectively. The design of *in vitro* cell differentiation assay served as an excellent investigation model for exploring a complex cellular and molecular mechanism in the pathogenesis of chronic diseases like OA, wherein appropriate animal models are not available in the current state. The findings of these assays showed that SF act like a 'niche' providing a microenvironment and essential protein-make up, which enable immune cells to be functionally active. Exact signalling mechanism between SF microenvironment and immune cell precursors during this process however needs further evaluation (Publication 4 and 5).
4. In the cell differentiation assays, a prominent increase in M2 phenotype after the induction of early grade SF samples (KL grade-II) indicated a possible natural buffering response offered by SF, which was seen declined in the later stages. An increase in the percentage of M1 phenotype after the SF induction with moderate to terminal grade OA samples (KL grade-III and IV) can be designated to insufficiency of the combating efforts to neutralize

the increasing inflammatory microenvironment and a possible switching of M2 macrophages to M1 phenotype (Publication 5).

5. Significantly higher M1/M2 ratios were estimated in the newly differentiated cells, when U937 cells were induced with SF of KL grade-III and IV during *in vitro* cell differentiation assays; these ratios further showed a positive correlation with KL score system, underling a potential of these ratios as a predictor of OA severity. Immunophenotyping of the SF cells of progressive OA grades, revealed the highest percentage of CD14+ cells in KL grade-II fluid samples; percentage of CD86+ and CD163+ cells were minimal (Publication 5).
6. Proteome analysis of different grades of OA SFs revealed significantly expressed proteins like Ig light chain proteins (kappa and lambda variable), free histones and neurotensin (a receptor of MRGPX2), which have an active role in mast cell maturation and activation. Also, significantly high levels of S100 proteins (S100A8/A9, S100A12, S1004 and S100P) were detected in SF samples of all OA grades; these proteins are closely linked with neutrophil and macrophages activation and transmigration. It is likely that high levels of these proteins contributed for enabling SF samples to induce immune cell differentiation as was evident in the cell differentiation assays (Publication 4 and 5).
7. RNA-seq analysis of osteophyte samples revealed an ongoing active bone and cartilage remodelling marked by a significant up-regulation of cathepsins (G and K), COL1A1, COL1A2, SELE and MMP-1, 3, 13. A significant up-regulation of genes like CMA1, CPA3, MS4A2/ FcERI (4.22-fold), IL1RL1 and PLA2G2A indicated an active involvement of mast cells, which was further confirmed by IHC study, wherein outnumbered anti-TPSAB1-stained cells and anti-FcERI-stained cell were found in the cartilaginous region and in the space between subchondral cancellous bone trabeculae in the osteophyte samples. A successful differentiation of HSC into FcERI+ after 9 days of SF treatment provided a strong base for a possibility that differentiation and invasion of the mast cells into osteophytes is mediated by SF, as proposed by the author (Publication 4).

Declaration of interests

All the authors declare that there is no conflict of interest.

SUMMARY IN ESTONIAN

Osteoartriidi patogenees: immunoloogiline kulg sünoviaalmembraan- sünoviaalvedelik teljel

Sissejuhatus

Osteoartriit (OA) on luu- ja lihaskonna haigus, mida iseloomustavad liigeste jäikus, turse, valu ja liikumisvõime kadu. See on eakate inimeste kõige levinum luu- ja lihaskonna haigus. 2019. aasta ülemaailmse haiguskoormuse uuringu (Global Burden of Disease Study – GBD) andmetel on OA maailmas haigena elatud aastate arvu (Years Lived with Disability – YLD) poolest 15. kohal, moodustades 2,2% kõigist maailma haigusseisunditest (2019. aastal 18,9 miljonit). Ülemaailmne OA levimus on üle 7% (528 miljonit inimest), millest põlveliigese OA on kõige sagedamini esinev haigustüüp, moodustades 365 miljonit juhtu kogu maailmas ning 61% haiguse tõttu kaotatud aastatest. Põlveliigese OA-le järgneb käte OA (142 miljonit juhtu, 24% OA kaotatud aastatest) ja seejärel puusaliigese OA (33 miljonit, 5,5% OA kaotatud aastaid). Luu- ja lihaskonna haiguste hulgas oli 2017. aastal enim haiguse tõttu kaotatud aastaid tingitud selja- ja kaelavaludest, millele järgneb OA, mis moodustab umbes 7,1% ülemaailmsest haiguskoormusest. Sotsiaalmajanduslikust vaatenurgast on OA eakate inimeste seas suur rahvatervise probleem, mis mõjutab üle 70% 65-aastastest elanikest ning on seotud paljude otseste tervisekuludega, nagu ambulatoorne- ja haiglaravi, sealhulgas liigeseasendusoperatsioonid, aga ka kaudsete kuludega, nagu haiguspäevad ja invaliidsus. Üldiselt moodustavad OA kulud 1–2% kogu maailma SKTst. Haiguse esinemissagedus suureneb elanikkonna vananemise ja rasvumise kui peamise OA-ga seotud riskiteguri suurenemisega. Arvestades OA põhjustatud märkimisväärset haiguskoormust avaldas International Osteoarthritis Research Society (OARSI) 2016. aastal sellekohase konsensusdokumendi ja tegevusjuhised.

Vaatamata sellele, et OA on üks kulukamaid ja invaliidsust tekitavamaid artriidivorme ning on levinum kui teised artriidid, ei ole siiani olemas OA patogeneetilist ravi ega isegi mooduseid selle progresseerumise tõhusaks peatamiseks, et parandada patsiendi elukvaliteeti ja leevendada ühiskonna majanduslikku koormust. Peamine raskus OA raviks mõeldud tõhusate ravimeetodite väljatöötamisel ilmneb haiguse heterogeensuses ja ebaselges patoloogias. Praegune teadustöö defineerib OA-d kui kergekujulist põletikulist haigust, mis mõjutab kogu liigest. OA näib olevat mehaaniliste, rakuliste ja süsteemsete molekulaarsete tegurite (k.a immunoregulatoorsed ja komplementvalgud) mitmetahulise koosmõju tulemus, millel on omakorda süsteemne roll seoses kroonilise põletikuga. Sünoviaalmembraan (SM) ja sünoviaalvedelik (SV) on olulised põletikukohad. Olles hästi ühendatud süsteemse vereringega, toimib SM vahendajana süsteemsete kahjustuste muutmisel haiguse spetsiifiliseks patoloogiaks. SV toimib SM mõjutuse ülekandjana, mis vahendab signaali-

ülekannet kõigi liigesekudede vahel ning tekitab haigusspetsiifilisi kahjustusi OA arengu varases staadiumis. Põletikuline SM on hüpertroofiline ja toodab põletikulisi ja kataboolseid mediaatoreid, nagu OA-spetsiifilised tsütokiinid, hapniku radikaale, lämmastikoksiidi (NO), prostaglandiin E2 (PGE2) ja neuropeptiide, mis muudavad raku metabolismi ja kõhre maatriksi metabolismi. Veelgi enam, põletikuline SM toodab ka liigselt kõhre lagunemist soodustavaid proteolüütilisi ensüüme. Kõhre lagunemissaadused indutseerivad omakorda SM põletikku, mis veelgi süvendab OA patsientide liigeste lagunemist ja kliinilisi sümptomeid. Kondrotsüütide ja sünoviotsüütide poolt toodetud põletikueelsed mediaatorid suurendavad oksüdatiivset stressi, mis põhjustavad samuti liigesekudede kahjustusi.

Arvestades põletiku võtmerolli OA patoloogias, arvati, et tavapäraste põletikuradade (nagu anti-TNF või anti-IL1β ained) süsteemne blokeerimine on tõhus ravi. Siiski leiti, et see ei ole efektiivne raske OA korral ja on väheefektiivne erosiivse OA korral. See rõhutab tõsiasja, et OA põletikulist mehhanismi ei ole täielikult selgitatud, mistõttu edasine uurimine on põhjendatud.

Käesoleva doktoritöö eesmärk oli hinnata OA põletikumehhanismi SM-SV teljel. Sellega seoses on SM rolli uuritud mikrotasandil alates geenisekspressiooni uuringutest kuni üksiku raku-RNA järjestamiseni. SV-analüüs piirdub siiski vedeliku molekulaarse koostise uurimisega ja selle funktsionaalse seotuse laiema selgitamisega haiguse patoloogias. *In vitro* rakkude diferentseerumise analüüsil kasutati erineva haiguse raskusastmega OA SV proove ning nende modulatsioonivastust uuriti põletikuliste ja immunoloogiliste parameetrite põhjal. Mõlema bioloogilise analüüsi eeliseks oli nende füsioloogiline sarnasus *in vivo* tingimustega; rakud indutseeriti SV proovidega, kasutades nende loomulikku patobioloogilist valgu koostist erinevates OA etappides ja seetõttu eeldati, et nad reageerivad sarnaselt *in vivo* tingimustele. Samal ajal viidi läbi SV-proovide proteoomika analüüs ja immuno-fenotüüpimine, et toetada bioloogiliste analüüside tulemusi. Viidi läbi ka SM rakugeenide ekspressiooni-uuringud, et kaardistada põletiku muster OA erinevates etappides. Osteofüüdid on OA liigeste lahutamatu osa; osteofüütide uuringud on aga fragmentaarsed, ebaselged ja nendega seotud molekulaarseid ja rakulisi protsesse pole piisavalt selgitatud, eriti OA patoloogia kontekstis. Autor täitis selle ülesande oma doktoritöös, et vähendada ebaselgust viies läbi täielikult formeerunud osteofüütide RNA-seq analüüsi. RNA-seq analüüs, millele järgnes geenisekspressiooni funktsionaalne analüüs, näitas, et immuunrakkude aktiivne osalemine ja SV roll immuunrakkudest diferentseerumisel osteofüütide küpsemisel on OA patoloogias uus teadmine. See info võib olla tulevikus aluseks diagnosika kavandamisel ja sobivate ravimeetodite määramisel.

Eesmärk ja ülesanded

Selle doktoritöö üldeesmärk oli OA patoloogias esinevasünooviumi-SF telje ümber koonduva põletikulise mehhanismi põhjalik hindamine. See hõlmas erinevate OA poolt mõjutatud koeproovide, nagu sünoviumi biopsiad ja SF

proovid, mis hõlmavad haiguse erinevat raskusastet ja osteofüüte, mitmetasandilist analüüsi. Üldeesmärgi täitmiseks kavandati katsete seeriad allpool kirjeldatud konkreetsete eesmärkidega:

1. Kirjeldada ja võrrelda peamiste põletikuliste vahendajate variatsioonimustrit OA loomuliku progresseerumise erinevatel etappidel (publikatsioonid 1 ja 3)
2. Teha kindlaks SF funktsionaalne osalus OA patogeneesis, arvestades selle potentsiaali põlveliigese õõnsuse põletikulist mikrokeskkonna loomisel (publikatsioonid 1, 2 ja 3)
3. Hinnata SF võimet indutseerida immuunrakkude diferentseerumist (publikatsioonid 4 ja 5)
4. Osteofüütide molekulaarsete ja rakuliste mehhanismide ning nende seoste selgitamine OA patoloogiaga (publikatsioon 4)

Materjal ja meetodid

Uuringusse kaasati patsiendid Tartu Ülikooli Kliinikumi ortopeediakliinikust ja Bharati Vidyapeethi ülikooli Bharati haiglast (Indias), kellel on kinnitatud põlveliigese OA diagnoos (vanuserühm 40-75 aastat). Väljaarvamiskriteeriumiks oli reumatoidartriit (RA), infektsioosne artriit, OA kaasuva kasvajalise haigusega, OA põlveliigese vigastusega vähem kui 2 aasta jooksul. OA diagnoositi kogenud reumatoloogi või ortopeedi poolt kliiniliste ja radiograafiliste tunnuste põhjal. Haiguse raskusaste määrati Kellgren-Lawrence'i (KL) radiograafilise skoori järgi. Kokku koguti OA erinevate staadiumidega patsientidelt 26 SM proovi (KL I st. = 6; KL II st = 6; KL III st = 6; KL IV st = 8) ja 32 SV proovi (8 SV proovi igast KL staadiumist). qRT-PCR analüüs viidi läbi, kasutades SM-ist eraldatud RNA-d; OA progresseerumise erinevates etappides hinnati põletikueelsete tegurite, nagu IL-1β, IL-15, PGE2 ja NGF variatsioonimustrit. Määrati OA SV funktsionaalne osalus haiguse patoloogias. Selleks kavandati diferentseerumise testid, mille käigus indutseeriti THP1 ja U937 rakke erineva KL klassi SV-ga 48 tunni jooksul ning rakukultuurisöötmes hinnati NO vabanemist. Kokku analüüsiti 12 proovi (igast klassist 3). Samuti hinnati rakualusest eraldatud RNA-d kasutades põletikueelsete markerite nagu MMP-1, 13 ja VEGF-1 geeniekspressiooni.

Makrofaagide ja nende tüüpide (tüüp M1 ja M2) määramiseks kasutati (12 SV proovi – 4 SV proovi igast II-IV KL staadiumist) immunofenotüpiseerimist antikeha paneelil – CD14 (pan-makrofaagi marker), CD86 (M1 tüübi marker) ja CD163 (M2 tüübi marker). Immuno-fenotüüpimine viidi läbi 24 tunni jooksul pärast vedeliku kogumist. *In vitro* rakkude diferentseerumise test viidi läbi U937, THP1 ja HSC-dega. U937 indutseeriti erinevate KL klasside SV-ga 48 tundi, samas kui THP1 ja HSC-sid töödeldi SV proovidega 9 päeva. Pärast SV inkubatsiooniperioodi eraldati äsja diferentseerunud rakud voolutsütomeetria abil. U937 rakkude jaoks kasutatud antikehade paneel sisaldas rakupinna markereid, nagu CD14, CD86 ja CD163. SF-iga töödeldud THP1 rakud värviti

HLA-DR ja CD206-ga, samas kui FcERI-d kasutati SF-iga töödeldud HSC-de värvimiseks. Lisaks uuriti U937 testiga saadud äsja diferentseerunud rakkude funktsionaalset seisundit tsütokiinide, nagu TNF-α, IL-6, IL-4, IL-10, IFN-γ ja GMCSF geeniekspressiooni abil. Proteoomi analüüs tehti 16-st SV proovist (4 SV proovi igast KL staadiumist), et uurida OA SV valgu olemust, mis juhivad või reguleerivad immuunrakkude diferentseerumisprotsessi, nagu nähti *in vitro* rakkude diferentseerumise analüüside tulemustes.

Osteofüütide RNA-seq analüüs tehti, et selgitada nendes esinevaid molekulaarseid ja rakulisi muutusi, mis on seotud OA patoloogiaga; selleks koguti kuuelt OA patsiendilt sääreluu mediaalselt kondüülilt osteofüüdid. Kontrollina kasutati sääreluu välimise kondüüli epifüüsi trabekulaarsest luust kogutud mitteosteosteofüütilist kude. RNA-seq analüüsi tulemused kinnitati, kasutades oluliselt ülesreguleeritud geenide, sealhulgas CMA1, TPSAB1, CPA3, MMP-1, MMP-3 ja MMP-13 qRT-PCR analüüsi. Lisaks kinnitati nuumrakkude olemasolu osteofüütide ja kontrollproovide immunohistokeemiaga, kasutades TPSAB1 ja FcERI vastu suunatud antikehi.

Peamised tulemused ja järeldused:

1. Sünoviumi geeniekspressiooni uuring tõi välja OA liigestes esineva põletiku püsiva olemuse ja tõstis veelgi esile põletikumarkerite erinevat ekspressiooni OA progresseerumise igas etapis. Näiteks olid peamised OA tsütokiinid (IL-1β ja IL-15) märkimisväärselt ekspresseeritud KL I astme biopsiates. Nende põletikuregulaatorite liigne esinemine varases staadiumis (kahtlane OA) annab tõenäoliselt biokeemilise vallandaja OA arengule isegi enne kindlate kliiniliste tunnuste ilmnemist. Teisisõnu, kõrgenenud IL-β ja IL-15 koosmõju OA varases staadiumis annab vajaliku tõuke kõhrekoe kiiremaks kadumiseks haiguse hilisemates staadiumides, nagu näitas autor oma varem avaldatud uuringus (Kulkarni et al., 2016). Teisest küljest näitas peamiste põletikueelsete tegurite hindamiseks tehtud SF-analüüs II ja III astme KL proovides oluliselt kõrgemat taset, mis korreleerus veelgi SF-i väljakutse testi tulemustega (publikatsioonid 1 ja 2).
2. SV uuringu tulemused näitasid selgelt, et OA SF-s esinevad põletikueelsed tegurid suutsid THP1 ja U937 rakkudes põletikku esile kutsuda. NO kõrgeimat taset hinnati pärast induktsiooni varase ja mõõduka astme (KL I/II/III aste) SF proovidega. Need tulemused on kooskõlas põletikueelse vahendaja tasemega SF proovides, samuti põletikueelsete markerite, sealhulgas MMP-1, MMP-13 ja VEGF-1 geeniekspressiooniga, mida hinnati SF-i indutseeritud THP1 ja U937 rakkudes. Oluline on see, et sünooviumi geeniekspressiooni uuringu ja SF-nakkuse testi tulemused (koos vaadeldes) näitavad OA KL II ja III astme silmapaistvate põletikuliste staadiumidena (publikatsioonid 1, 2 ja 3).
3. OA SF indutseeritud U937, THP1 ja HSC-d erinevatel ajahetkedel näitasid selget diferentseerumist vastavalt efektormakrofaagideks ja nuumrakkudeks. Sedatüüpi analüüse võib pidada suurepäraseks uurimismudeliks krooniliste haiguste, nagu OA, patogeneesi käigus kujuneva keeruka rakulise ja

molekulaarse mehhanismi uurimiseks, ja seda kontekstis, kus sobivad loommudelid pole hetkel saadaval. Nende analüüside tulemused näitasid, et SF toimib nagu "nišš", mis tagab mikrokeskkonna ja olulise valgulise kontesti, mis võimaldab immuunrakkudel olla funktsionaalselt aktiivsed. Täpne signalisatsioonimehhanism SF mikrokeskkonna ja immuunrakkude prekursorite vahel selle protsessi ajal vajab siiski täiendavat hindamist (väljaanded 4 ja 5).

4. Rakkude diferentseerumise testides näitas rakkude populatsioonis M2 fenotüübi märgatav tõus pärast varajase astme SF proovide (KL II aste) esilekutsumist SF võimalikule loomulikule puhverdusreaktsioonile, mis hilisemates etappides oli vähenenud. M1 fenotüübi protsendi suurenemist täheldati pärast SF indutseerimist mõõduka kuni terminaalse astme OA proovidega (KL III ja IV aste) ning seda võib pidada ebapiisavaks võitluseks kasvava põletikulise mikrokeskkonna neutraliseerimiseks ja võimalikuks ümberlülitamis-ekspolariseeritud makrofaagide ühest fenotüübist (väljaanne 5).
5. Oluliselt kõrgemad M1/M2 suhted hinnati äsja diferentseerunud rakkudes, kui U937 rakke indutseeriti in vitro analüüside käigus KL III ja IV astme SF-ga; need suhted näitasid lisaks positiivset korrelatsiooni KL skoorisüsteemiga, rõhutades nende suhtarvude potentsiaali OA raskusastme ennustajana. Progresseeruva OA astme SF-rakkude immunofenotüpiseerimine näitas KL II astme vedelikuproovides suurimat CD14+ rakkude protsenti; CD86+ ja CD163+ rakkude protsent oli minimaalne (väljaanne 5).
6. Erinevate OA SF-de proteoomianalüüs näitas märkimisväärselt ekspresseeritud valke nagu Ig kerge ahela valgud (kappa ja lambda variaablid), vabad histoonid ja neurotensiin (MRGPX2 retseptor), millel on aktiivne roll nuumrakkude küpsemisel ja aktivatsioonil. Samuti tuvastati kõigi OA klasside SF proovides oluliselt kõrge S100 valkude (S100A8/A9, S100A12, S1004 ja S100P) sisaldus. Need valgud on tihedalt seotud neutrofiilide ja makrofaagide aktivatsiooni ja transmigratsiooniga. On tõenäoline, et nende valkude kõrge tase võimaldas SF-proovidel indutseerida immuunrakkude diferentseerumist, nagu ilmnes rakkude diferentseerumise analüüsides (väljaanded 4 ja 5).
7. Osteofüütide proovidest teostatud RNA-seq analüüs näitas käimasolevat aktiivset luu ja kõhre ümberkujunemist, mida iseloomustab katepsiinide (G ja K), COL1A1, COL1A2, SELE ja MMP-1, 3, 13 oluline ülesreguleerimine. Geenide, nagu CMA1, CPA3, MS4A2/FcERI IL1RL1 ja PLA2G2A reguleerimine näitas nuumrakkude aktiivset osalust. Sedakinnitas veelgi IHC uuring, kus anti-TPSAB1 värvitud rakud ja anti-FcERI värvitud rakud olid arvulises ülehulgas justkõhrepiirkonnast ja subkondraalsete käsn-luutrabekulide vahelisest ruumist võetud osteofüütide proovides. HSC edukas diferentseerumine FcERI+-ks pärast 9-päevast SF-ravi võib viidata võimalusele, et nuumrakkude diferentseerumist ja invasiooni osteofüütideks vahendab SF, nagu autor on pakkunud (väljaanne 4).

SUMMARY IN MARATHI

संधिगतवात (osteoarthritis) ही वृद्धापकाळातील सर्वसामान्य व्याधी असुन ह्यावर प्रस्तुत काळात शाश्त्रक्रिये शिवाय कोणतेही उपचार उपलब्ध नाहीत. ह्या व्याधीची स्वभाव विषमता आणि संदिग्ध संप्राप्ती ही संधिवातावर प्रभावी चिकित्सा पद्धती विकसित करण्यातील प्रमुख आव्हाने आहेत. पारंपारिकपणे 'वृध्दत्वावस्थेतील व्याधी' म्हणून ओळखला जाणारा, संधिगतवात हा प्रस्तुत काळात एक जटिल आणि कष्टसाध्य व्याधी म्हणून स्वीकारला गेला असुन, व्याधी-संप्राप्तीमध्ये मुख्य: संधी-ऊतकांत असणाऱ्या जीर्ण विदहाचा (chronic inflammation) समावेश आढळून आला आहे. व्याधी-संप्राप्तीमधील संदिग्धता दूर करण्यासाठी ह्या मुद्यावर (जीर्ण विदाह) व्यापक विश्लेषणाची गरज व्यक्त करण्यात आली आहे.

प्रस्तुत प्रबंधाचे मुळ उद्दिष्ट हे, संधी-ऊति आणि संधी-श्लेष ह्या अक्षाभोवती (synovium-synovial fluid axis) आवर्तित जीर्ण-विदाह संप्राप्तीचे सखोल विश्लेषण करणे असे आहे. आमच्या हे लक्षात आले आहे की, संधी-ऊति आणि संधी-श्लेष ही जानू संधीमधील प्रमुख विदाह स्थाने आहेत. आज पर्यंत, संधिगत वाताच्या व्याधी संप्राप्तीमध्ये जानू संधी-श्लेषाचा सहभाग हा केवळ त्या श्लेषाची संरचना समजून घेण्यापुरता मर्यादित राहीला असुन, ह्या श्लेषाचा संप्राप्तीमध्ये असणाऱ्या कार्यात्मक सहभागाचे (functional involvement) संशोधन करण्यास विस्तृत वाव आहे. प्रस्तुत प्रबंधातून संधी-श्लेषाच्या कार्यात्मक सहभागाचे घनाणु आणि कोशिका स्तरांवरील संकेतांचे विग्रहण (decoding of molecular and cellular signalling) करण्याचा प्रयत्न केला गेला आहे.

प्रस्तुत प्रबंधामध्ये संधिगत वातग्रस्त ऊतिका, जसे संधी-ऊति, संधी-श्लेष आणि अस्थी-ग्रंथी यांचे बहुस्तरीय विश्लेषण केले गेले. त्याचप्रमाणे व्यावसायिक तत्वावर उपलब्ध असणाऱ्या मानवी कोशिका (THP1 and U937 cell lines) आणि हेमॅटोपोएटिक मूळ कोशिका (hematopoietic stem cells) वापरून कृत्रिम परिवेशीय (in vitro) प्रयोग करण्यात आले. या प्रयोगांतून मिळालेल्या निष्कर्षांना अधिक पुष्टी देण्यासाठी संधी-श्लेषाचे प्रोटीओम विश्लेषण आणि इम्युनोफेनोटाइपिंग केले गेले. संधी-ऊति जनुक अभिव्यक्ती प्रयोगांनी संधिगत वात व्याधी संप्राप्तीमध्ये 'सतत स्वरूपात उपस्थित असणारा विदाह' प्रमाणित करण्यात आला. संधिगत वात व्याधी संप्राप्तिच्या प्राथमिक अवस्थेत अधिक प्रमाणात असणारा विदाह हा बव्हंशी ह्या व्याधीच्या पुढील अवस्थांमध्ये वेगाने होणाऱ्या कुर्चास्थि-हानीला (cartilage-loss) उद्दीपित करतो. तसेच कृत्रिम परिवेशीय प्रयोगांमध्ये, ह्या व्याधीच्या विविध अवस्थांमधील रुग्णांच्या जानू-संधीतील संधी-श्लेषामध्ये मानवी कोशिकांना वाढविल्यास, हा संधी-श्लेष कोशिकांमध्ये विदाह उत्पन्न करण्यास सक्षम असल्याचे आढळून आले. ह्याच कृत्रिम परिवेशीय प्रायोगिक संरचनेचा वापर करून आम्ही असे सिद्ध करू शकलो की, संधिगत वातामध्ये जानू-संधीतील संधी-श्लेष हा रोगप्रतिकारक कोशिकांमध्ये अवस्थांतरण (immune cell differentiation) घडवून आणण्यास सक्षम असुन हा श्लेष, व्याधी संप्राप्तीमध्ये होणाऱ्या अश्या अवस्थांतरणासाठी अत्यावश्यक अशी सूक्ष्म-परीस्थिती (microenvironment) प्रदान करतो. कृपया हे लक्षात घ्या कि, रोगप्रतिकारक कोशिकांमध्ये होणारे हे अवस्थांतरण संधिगत वात व्याधी संप्राप्तीमध्ये विदाह उत्पन्न करण्यास आणि त्याचे निर्वहन करण्यास कारणीभूत आहे. संधिगत वातामधील संधी-श्लेषाचे प्रोटीओम विश्लेषण हे आमच्या वरील निष्कर्षास दृढता प्रदान करते. ह्या प्रोटीओम विश्लेषणात अनेक अशी प्रथिने अत्याधिक मात्रेत आढळून आली आहेत जी रोगप्रतिकारक कोशिकांमध्ये अवस्थांतरण करण्यास कारणीभूत आहेत. संधिगत वात व्याधी संप्राप्तीच्या दृष्टीकोनातून अस्थि-ग्रंथीतील घनाणु व कोशिका स्तरांवरील संकेतांचे विग्रहण करण्यासाठी अस्थि-ग्रंथी नमुन्यांचे

RNA-seq विश्लेषण करण्यात आले. ह्या प्रयोगाचे कार्यात्मक विश्लेषण असे दर्शवते की, अस्थि-ग्रंथीमध्ये अस्थी धातू पुनर्निर्माणाची प्रक्रिया सतत गतिमान असुन त्यात मास्ट-कोशिकांचा (mast cells) क्रियाशील सहभाग दिसून आला आहे. प्रस्तुत प्रबंधाद्वारे अस्थि-ग्रंथीमधील घनाणु व कोशिका स्तरांवरील संकेतांमध्ये मास्ट-कोशिकांचा (mast cells) कार्यात्मक सहभाग हा प्रथमच दर्शवला गेला आहे. संधिवातावर प्रभावी चिकित्सा पद्धती विकसित करताना प्रस्तुत प्रबंधाद्वारे नोंदवली गेलेली निरीक्षणे जरूर विचारात घेतली जातील असा विश्वास ह्या प्रबंधाची लेखिका व्यक्त करते.

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APPENDIX

Table 1: A list of significantly up-regulated and down-regulated genes in the osteophyte samples with their logFC and P-values

Up-regulated Genes		
Gene Name	**LogFC**	**P-value**
CPA3	4.027533	5.09E-18
SELE	2.536014	2.14E-16
MS4A2	4.220766	3.78E-15
PLA2G2A	4.644088	5.87E-12
CSN1S1	5.942081	1.44E-10
HAPLN1	4.354046	1.48E-10
GABRA4	3.303431	4.35E-10
PRG4	2.714763	5.14E-10
THBS4	2.993005	6.09E-10
IBSP	2.006641	7.31E-10
SLC36A2	2.247509	7.09E-09
HPGD	3.213818	9.49E-09
OGN	2.890213	4.86E-08
ASPN	2.432588	6.50E-08
CTSG	6.380478	7.17E-08
F5	2.485848	1.88E-07
FAM38B	2.125555	4.49E-07
IL1RL1	2.520738	8.72E-07
ZIC1	4.85166	1.12E-06
PRSS35	2.469275	1.73E-06
STMN2	2.835148	1.70E-06
MMP-13	3.19846	2.01E-06
CRTAC1	2.740265	2.06E-06
SHOX2	2.027773	7.05E-06
ACP5	2.83225	7.15E-06
COMP	3.324406	9.58E-06
COL1A2	2.01073	1.29E-05
LRFN5	3.088649	1.65E-05
TMEM196	2.776174	1.94E-05
HBA2	2.960499	2.08E-05
CTSK	2.240189	2.15E-05
CKB	2.25553	2.41E-05
GNG4	2.377001	3.22E-05
ADCYAP1	2.582899	3.33E-05
CNR1	2.254559	5.24E-05
ST18	2.889322	5.83E-05
MMP3	3.539804	6.33E-05
FZD10	2.231317	0.000133
GJB2	3.455437	0.000135
CYP27C1	4.37209	0.000173
C1orf186	3.272109	0.000199
HDC	2.110747	0.000243
CMA1	5.007723	0.000274
COL1A1	2.038679	0.000368
SYT6	2.129815	0.000422

Up-regulated Genes		
Gene Name	**LogFC**	**P-value**
AMPH	2.072226	0.000523
AMTN	3.814246	0.000729
TNFSF11	2.405489	0.001057
GALNT14	4.471982	0.001105
TRIM11	2.051431	0.001203
NELL1	4.426061	0.001209
TIMD4	2.79137	0.001212
HHIP	3.320885	0.001394
MRGPRX2	4.517302	0.001406
GFPT2	2.213461	0.001616
NPHS1	3.499433	0.001877
RASL12	2.011284	0.002044
HEMGN	2.173148	0.002365
CXCL9	2.226117	0.002528
IDO1	3.382825	0.002929
MYO3A	2.28746	0.00321
ADAM23	3.193211	0.003252
LRRC8E	2.234257	0.003983
SERPINA5	2.802678	0.004112
GYPA	2.738816	0.004161
SLC4A1	4.885617	0.004197
LIF	3.908902	0.004473
OPCML	2.428177	0.004786
PART1	2.41668	0.004784
CXCL10	2.225288	0.006315
FAM40B	2.022329	0.00656
C21orf37	4.084623	0.006829
WFDC1	2.136605	0.006992
KIF4A	2.248998	0.007723
FAM133A	2.053699	0.008372
CLDN10	2.516074	0.009417
SLC9A2	2.165385	0.010866
TUBA8	2.401238	0.010872
CXCL11	2.190709	0.013103
SULT1B1	3.152541	0.013765
MMP1	3.027436	0.014302
CILP	2.148754	0.014409
CRLF1	3.817562	0.015141
CA1	2.451945	0.017428
MARCO	3.779762	0.018039
LINGO1	2.318577	0.018117
C10orf105	2.176865	0.0222
Down-regulated Genes		
Gene Name	**LogFC**	**P-value**
APOB	-2.03918	9.05E-11
CADM2	-2.95074	1.41E-08
TMEFF2	-4.15435	0.000413
GNAZ	-3.41417	0.000625
GABRA2	-2.77263	0.000654
NRG3	-4.14112	0.001502

Down-regulated Genes		
Gene Name	**LogFC**	**P-value**
CHIT1	-3.25043	0.003048
C6orf10	-3.72459	0.003143
NETO1	-2.91119	0.00346
FAM86DP	-3.6999	0.00405
LOC100129345	-3.6983	0.004298
GLIS1	-3.69735	0.004474
IGSF1	-2.84473	0.004914
TRAF2	-3.68328	0.006547
SERPINA12	-2.98392	0.007455
TINAGL1	-3.55897	0.007825
LOC148824	-3.56945	0.009136
C1orf74	-3.55768	0.009161
FLJ30403	-3.40428	0.015644
LOC653113	-3.43474	0.018687
PIAS4	-3.40745	0.020407
PRINS	-3.40161	0.021045

Table 2: A grade-wise demonstration of key proteins found in the proteome analysis of OA SFs and are associated with mast cells regulation

Protein Name	Protein Abbreviation	LogFCG2G1	LogFCG3G1	LogFCG4G1	Ref
Immunoglobulin lambda variable 2-18	IGLV2-18	37.5896545	NA	37.42546	
Immunoglobulin lambda variable 3-1	IGLV3-1	36.59979569	NA	NA	
Immunoglobulin kappa variable 2-40	IGKV2-40	37.05100621	41.67871	37.29651	
Immunoglobulin lambda variable 2-11	IGLV2-11	2.431556072	2.782218	NA	
Immunoglobulin superfamily containing leucine-rich repeat protein	ISLR	NA	39.41972	NA	
Immunoglobulin kappa variable 1-13	IGKV1-13	NA	37.90953	NA	
Immunoglobulin kappa variable 6-21	IGKV6-21	NA	36.9165	NA	
Immunoglobulin lambda variable 4-69	IGLV4-69	NA	2.773235	NA	
Immunoglobulin superfamily containing leucine-rich repeat protein	ISLR	NA	NA	41.52395	
Immunoglobulin lambda variable 1-44	IGLV1-44	NA	NA	36.97934	
Immunoglobulin kappa variable 1-13	IGKV1-13	NA	NA	36.86832	
Immunoglobulin lambda variable 4-69	IGLV4-69	NA	NA	2.380682	
Protein S100 (Fragment)	S100A6	41.16882	40.40297	42.1267	
Protein S100-A8	S100A8	38.80698	43.95978	43.62165	
Protein S100-A9	S100A9	38.33136	44.35842	43.4431	
Protein S100-A4	S100A4	3.127559	-37.9506	2.521137	
Protein S100-A11	S100A11	0.747533	3.450775	4.473638	
Protein S100-A12	S100A12	NA	43.6355	44.17231	
Protein S100-P	S100P	NA	42.32842	41.86717	
Histone H3.1	H3C1	42.15141	43.86723	42.13659	
Histone H1.2	H1-2	40.8021	NA	40.73825	
Isoform 1 of Core histone macro-H2A.1	MACROH2A1	40.35853	41.30319	40.28084	
Histone H2A type 2-B	H2AC21	38.82677	40.98707	NA	
Histone H2A type 2-C	H2AC20	37.47739	38.57285	36.1769	
Histone H1.10	H1-10	36.92155	36.63726	36.05325	
Histone H1.5	H1-5	4.143112	1.894718	3.143245	
Histone H4	H4C1	4.135902	5.062983	3.918993	
Histone H3.2	H3C15	3.952981	4.763897	3.338169	
Histone H2A	H2AZ2	3.513314	38.20615	4.071692	

Protein Name	Protein Abbreviation	LogFCG2G1	LogFCG3G1	LogFCG4G1	Ref
Histone H2B	H2BC15	2.290555	4.26651	2.707146	
Methionine aminopeptidase 2	METAP2	35.78272	33.33631	35.87134	
Xaa-Pro aminopeptidase 1	XPNPEP1	34.63523	37.59244	NA	
Aminopeptidase B	RNPEP	2.703409	5.132456	5.127756	
Alpha-actinin-1	ACTN1	40.01584	44.90512	44.6893	
Actin-related protein 2/3 complex subunit 3	ARPC3	39.73583	42.46596	42.05718	
Actin-related protein 3	ACTR3	2.494164	4.897881	4.0529	
Alpha-actinin-4	ACTN4	2.359276	4.658768	4.330454	
Actin-related protein 2	ACTR2	2.279216	4.53598	3.858559	
Actin, cytoplasmic 1	ACTB	2.240241	-37.5188	2.776209	(Kormelink et al., 2016)
Beta-actin-like protein 2	ACTBL2	NA	37.29973	35.91144	(Kormelink et al., 2016)
Platelet-activating factor acetylhydrolase IB subunit beta (Fragment)	PAFAH1B2	36.83481	35.17532	NA	
Ras-related protein Rab-7a	RAB7A	37.22036	39.44037	38.90048	(Kormelink et al., 2016)
Heat shock cognate 71 kDa protein	HSPA8	1.609628	1.574592	1.596362	(Kormelink et al., 2016)
Filamin-A	FLNA	3.974561	4.215035	3.994411	
Carboxypeptidase	CTSA	35.9543	NA	35.5287	
Carboxypeptidase Q	CPQ	1.294259	2.359289	-36.5255	
Cathepsin L1	CTSL	37.15384	36.42123	NA	
Cathepsin D	CTSD	2.680759	1.436841	3.760144	
Cathepsin G	CTSG	-37.5698	5.721166	5.100312	
Dual specificity mitogen-activated protein kinase kinase 1	MAP2K1	38.16372	38.24167	36.92404	
Mitogen-activated protein kinase 1	MAPK14	NA	39.43511	39.30365	
Mitogen-activated protein kinase 14	MAPK14	NA	39.48305	38.15754	
Mitogen-activated protein kinase	MAPK3	NA	36.16989	36.2431	
Phospholipase A2	PLA2G2A	2.003637	2.129905	0.998775	

Table 3: A grade-wise demonstration of key proteins found in the proteome analysis of OA SFs and are associated with macrophages regulation

No.	Protein	Action	Fold change		
			G1-G2	G1-G3	G1-G4
1	Macrophage migration inhibitory factor (MIF)	Prevent random migration and stimulate population growth of macrophages	-0.2950	-0.9350	-38.6789
2	Macrophage-capping protein, (CAPG)	Regulate cell migration through actin fiber modulation	0	38.6234	41.3961
3	Matrix metalloproteinase-9, (MMP9)	Multiple roles including activation of macrophages	0	40.9872	41.6936
4	Prostaglandin E synthase 3, (PTGES3)	Regulate release of inflammatory prostaglandin by macrophages	37.9651	37.1098	0
5	Osteopontin C	Osteopontin affects macrophage polarization promoting endocytic but not inflammation (Liu et al., 2022)	0	0	35.9557
6	Tumor necrosis factor alpha-induced protein 8 (GCA)	Inflammatory response and immune homeostasis	0	35.5756	38.0931
7	Ras-related protein Rab-1A, RAB1A	Plays a role in cell adhesion and cell migration	0	40.6271	39.5567
8	Ras-related protein Rab-7a, RAB7A	Enables Phagocytosis	37.2203	39.4403	38.9004
9	Ras-related protein Rab-10 RAB10	Phagosome maturation	0	38.6868	38.1715
10	Ras-related protein Rab-8B (RAB8B)	antigen processing and presentation	1	39.6188	37.8691
11	Ras-related protein Rab-31 (RAB31)	serves essential roles in vesicle and granule targeting (Wu et al., 2020)	0	37.3470	37.2641
12	Ras-related protein Rab-14 (RAB14)	Rab14 had been found to play a crucial role in phagocytosis, homotypic phagosome and lysosome fusion (Schmeichel and Bisell, 2003)	0	37.7317	37.1225
13	Ras-related protein Rab-2A (RAB2A)	Ras-related GTP-binding proteins involved in the regulation of secretion (Hong et al., 2011)	0	39.6762	36.7864
14	Ras-related protein Rab-1B (RAB1B)	Regulates vesicular transport between the endoplasmic reticulum and successive Golgi compartments	0	35.7568	35.9216
15	Ras-related protein Rab-3D (RAB3D)	Involved in regulated exocytosis (Greening et al., 2016)	0	38.2553	35.2364
16	Ras-related protein Rab-5C (RAB5C)	endocytosis (Jin et al., 2014)	36.7335	38.2535	35.1564
17	Ras-related protein Rab-21 (RAB21)	Regulates integrin internalization and recycling, may regulate cell adhesion and migration (Macur et al., 2021)	0	39.1358	0

Table 4: Demographic details of OA patients included in the RNA-seq analysis of the osteophytes

Patient No.	Demographic details
Patient 1	Age: 76 years Sex: male BMI: 23.78 KL grade: 4
Patient 2	Age: 67 years Sex: female BMI: 35 KL grade: 4
Patient 3	Age: 69 years Sex: male BMI: 27.7 KL grade: 4
Patient 4	Age: 73 years Sex: female BMI: 32.4 KL grade: 4
Patient 5	Age: 52 years Sex: female BMI: 35 KL grade: 3
Patient 6	Age: 71 years Sex: female BMI: 29.1 KL grade: 4

Table 5: an overview of data set of SF samples used for the estimation of pro-inflammatory factors with their KL grades and statistical analysis

Patient No	KL grade	IL-1β	NO	NN
Patient 7	I	35.00±0.5	17.35±0.9	24.32±1.0
Patient 8	I	38.18	20.00±0.0	20.94±0.4
Patient 9	I	138.18±12.7	28.24±0.6	30.76±1.1
Patient 10	I	-	41.18±1.8	31.20±0.7
Patient 6	I	47.27 ± 4.5	14.70 ± 0.6	23.56 ± 0.0
	AVG	59.36 ± 39.62	24.29 ±9.59	26.16 ±4.09
Patient 4	II	2184.85 ± 10.5	11.47 ± 1.4	66.54 ± 1.9
Patient 5	II	642.42 ± 13.8	80.00 ± 7.6	82.68 ± 1.9
Patient 11	II	184.55±7.3	42.65±0.9	34.5±2.7
Patient 12	II	132.27±5.0	57.06±1.8	30.3±2.2
Patient 13	II	47.27±4.5	28.82±0.6	31.6±3.6
	AVG	638.27 ±800.39	44 ±23.48	49.12 ±21.47
Patient 3	III	603.03 ± 12.7	42.35 ± 7.7	42.98 ± 1.0
Patient 14	III	754.55±18.2	56.18±2.6	31.64±2.0
Patient 15	III	114.09±5.9	30.59±3.5	28.52±0.5
Patient 16	III	78.64±12.3	34.41±0.3	29.7±3.9
Patient 17	III	-	25.15±1.0	25.00±0.6
	AVG	430.67 ±278.73	37.74 ± 79	31.57 ±6.10
Patient 1	IV	23.64 ± 1.8	69.11 ± 15.4	24.65 ± 0.7
Patient 2	IV	22.27 ± 7.7	62.36 ± 0.6	77.66 ± 2.6
Patient 18	IV	41.36±4.1	25.29±1.2	67.85±0.2
Patient 19	IV	23.18±9.5	22.06±0.9	74.18±2.2
Patient 20	IV	36.82±2.3	22.4±0.0	74.40±6.8
	AVG	29.45 ±8.00	40.24 ±20.95	63.75 ±19.80
KL grade-wise statistical Analysis				
IL-1β	KL-I and II – ns; KL-I and III – ns; KL-I and IV – ns; KL-II and III – ns; KL-II and IV – ns; KL-III and IV – ns			
NO	KL-I and II – ns; KL-I and III – ns; KL-I and IV – ns; KL-II and III – ns; KL-II and IV – ns; KL-III and IV – ns			
NN	KL-I and II – ns; KL-I and III – ns; KL-I and IV – **; KL-II and III – ns; KL-II and IV – ns; KL-III and IV – *			
* $P < 0.05$, ** $P < 0.01$				

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PUBLICATIONS

CURRICULUM VITAE

Name: Priya Gajanan Kulkarni
Date: June 2, 1981, India
Citizenship: Indian
Address: University of Tartu, Faculty of medicine,
Institute of Clinical Medicine,
Department of Traumatology and Orthopedics
8, Ludvig Puusepa, Tartu, 50406, Estonia
Telephone: +372 5556 8685
Email: pgkulkarni2610@gmail.com; priya.kulkarni@ut.ee

Education:

- 2010 Master of Biomedical Science (MSc), Middlesex University, London, UK
- 2006 Masters of Surgery (MS) (Ayurved), University of Pune, India
- 2003 Bachelor of Ayurved Medicine and Surgery (BAMS), Maharashtra University of Health Sciences, Nasik, India

Professional Employment:

- ❖ Feb 2018 – present: pursuing PhD at Department of Pathophysiology, Institute of Biomedicine and Translational medicine, University of Tartu, Estonia
- ❖ Jan 2017 – May 2019: Assistant Professor, IRSHA, Bharati Vidyapeeth University, India
- ❖ June 2016 – Dec 2016: Senior Research Fellow, IRSHA, India
- ❖ March 2014–May 2016: Research fellow, IRSHA, India
- ❖ 2012–Feb 2014: Project Assistant, Interactive Research School for Health Affairs (IRSHA), Bharati Vidyapeeth University, Pune, India

Scientific work:

Field: osteoarthritis, inflammation, herbal pharmaceuticals

Publications:

1. Kulkarni P, Harsulkar A, Martson AG, Suutre S, Martson A, Koks S. Mast cells differentiated in synovial fluid and resident in osteophytes exalt the inflammatory pathology in osteoarthritis. *Int. J. Mol. Sci*. 2022, 23 (1), 541.
2. Ingale D, Kulkarni P, Electricwala A, Moghe A, Kamyab S, Jagtap S, Martson A, Koks S, Harsulkar A. *Genes*. 2021, 12 (7), 989.
3. Kulkarni P, Martson A, Vidya R, Chitnavis S, Harsulkar A. Pathophysiological landscape of osteoarthritis. *Adv. Clin. Chem*. 2021, 100, 38–90.
4. Ingale D, Kulkarni P, Koppikar S, Harsulkar A, Moghe A, Jagtap S: Reduced synovial inflammation and inhibition of Matrix metalloproteinases explicates anti-osteoarthritis activity of polyherbal formulations. *Indian. J. Pharmacol*. 2018, 50 (1), 22–29.

5. Kulkarni P, Deshpande S, Koppikar S, Patil S, Ingale D, Harsulkar A. Glycosaminoglycan measured from synovial fluid serves as a useful indicator for progression of osteoarthritis and complements Kellgren-Lawrence score. *BBA*. *Clin*. 2016, 6, 1–4.
6. Koppikar S, Kulkarni P, Ingale D, Shinde D, Wagh N, Deshpande S, Moghe A, Ranjekar P, Harsulkar A. Inflammatory response of cultured rat synoviocytes challenged with synovial fluid from Osteoarthritis patients correlates with their radiographic grading: A pilot study. *In*. *Vitro*. *Cell*. *Dev*. *Biol*. *Anim*. 2015, 51 (8), 843–850.
7. Kulkarni P, Koppikar S, Deshpande S, Wagh N, Harsulkar A. Meniscal tear as potential steering factor for inflammation may aggravate arthritis: two case reports. *J*. *Med*. *Case*. *Rep*. 2014, 8 (1), 1–4.

ELULOOKIRIJELDUS

Nimi: Priya Kulkarni
Sünniaeg: 2. juuni 1981, India
Kodakondsus: Indialane
Aadress: Tartu Ülikool, Meditsiini valdkond,
Kliinilise Meditsiini Instituut,
Traumatoloogia ja Ortopeedia Kliinik
8, Ludvig Puusepa, Tartu, 50406, Eesti
Telefon: +372 5556 8685
E-mail: pgkulkarni2610@gmail.com; priya.kulkarni@ut.ee

Haridus:

- 2009 biomeditsiiniteaduse magister (MSc), Middlesexi Ülikool, London, Ühendkuningriik
- 2007 kirurgia magister (MS) (Ayurved), Pune Ülikool, India
- 2002 Ayurveda meditsiini ja kirurgia bakalaureusekraad (BAMS), Maharashtra Terviseteaduste Ülikool, Nasik, India

Teenistuskäik:

- ❖ Veebruar 2018 – Mai 2022: doktorant Tartu Ülikooli Biomeditsiini ja translatsioonimeditsiini instituudi patofüsioloogia osakonnas; Doktoritöö esitatakse teemal "Osteoartriidi patogenees: immunoloogiline kulg sünoviaalmembraan – sünoviaalvedelik teljel" kaitsmine on planeeritud 2022. aasta oktoobris
- ❖ Jaanuar 2017 – Mai 2019: assistent, IRSHA, Bharati Vidyapeethi Ülikool, India
- ❖ Juuni 2016 – Detsember 2016: vanemteadur, IRSHA, India
- ❖ Märts 2014 – Mai 2016: teadur, IRSHA, India
- ❖ Mai 2012 – Veebruar 2014: projektiassistent, Tervise Uurimise Interaktiivne kool (IRSHA), Bharati Vidyapeethi Ülikool, Pune, India

Teaduslik ja uurimistegevus:
Eriala: Osteoartriit ja põletik

Publikatsioonid:

1. P. Kulkarni, V. Srivastava, K. Tootsi, A. Electricwala, A. Kharat, R. Bhonde, S. Koks, A. Martson and A. Harsulkar. Synovial fluid in knee osteoarthritis extends proinflammatory niche for macrophage polarization. *Mediat*. *Inflamm*. 2022 (submitted).
2. Kulkarni P, Harsulkar A, Martson AG, Suutre S, Martson A, Koks S. Mast cells differentiated in synovial fluid and resident in osteophytes exalt the inflammatory pathology in osteoarthritis. *Int*. *J*. *Mol*. *Sci*. 2022, 23 (1), 541.

3. Ingale D, Kulkarni P, Electricwala A, Moghe A, Kamyab S, Jagtap S, Martson A, Koks S, Harsulkar A. Synovium-synovial fluid axis in osteoarthritis pathology: A key regulator of the cartilage degradation process. *Genes*. 2021, 12 (7), 989.
4. Kulkarni P, Martson A, Vidya R, Chitnavis S, Harsulkar A. Pathophysiological landscape of osteoarthritis. *Adv*. *Clin*. *Chem*. 2021, 100, 38–90.
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