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Transcriptome profile of extracellular vesicles derived from the synovial fluid and blood of osteoarthritis patients

Bachelors Thesis

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INFOLEHT

Transcriptome profile of extracellular vesicles derived from the synovial fluid and blood of osteoarthritis patients

Osteoarthritis (OA) is a chronic, musculoskeletal disease that affects quite a sizeable portion of the older generation. Late-stage OA has a considerable negative impact on the quality of life; however, it is very difficult to diagnose OA in the early stages. Therefore, there is a need to find new non-invasive diagnostic methods for early disease detection. Extracellular vesicles (EVs) are capable of protecting their RNA cargo during transportation and for this reason, there is a growing interest in EV-RNAs as potential biomarkers for different diseases and conditions. miRNAs have been shown to be dysregulated in the synovial fluid (SF) of OA patients and have been presented as potential biomarkers. miRNAs such as miR-146a and miR-26b have been shown to play a potential role in OA pathogenesis and have also been found to be significantly dysregulated in OA-SF. However, as there is a lack of research done on blood-EVs, further experiments need to be done in order to determine if the SF-EV profile reflects in blood-EVs.

Keywords: extracellular vesicles; osteoarthritis; miRNA; blood; synovial fluid.

CERCS: B220 Genetics, cytogenetics

Ekstratsellulaarsete vesiikulite transkriptoomiline profiil osteoartriiti põdevate patsientide sünoviaalvedelikus ja veres

Osteoartriit (OA) on krooniline luu- ja lihakonna haigus, mis mõjutab üsna suurt osa vanemaealisest elanikkonnast. OA-d on raske varajases staadiumis diagnoosida mistõttu otsitakse lahendusi, kuidas mitteinvasiivselt diagnoosida varajases staadiumis OA-d ning selle kaudu parandada OA põdevate patsientide elukvaliteeti. Ekstratsellulaarsed vesiikulid (EV-d) on võimelised RNA-d kaitsma transpordi ajal ning selletõttu EV-de RNA-d pakuvad huvi kui biomarkerid diagnostikas. miRNA-d on näidatud, et OA patsientide sünoviaalvedelikus (SF) on nende ekspressioonimuster muutnud ja seetõttu võiks need olla potentsiaalseks biomarkeriteks. miRNA-d nagu miR-146a ja miR-26b võtavad osa OA progressioonis ning on leitud, et need on oluliselt dereguleeritud nii OA-SF kui ka OA-SF EV-des. Veres olevaid EV-sid on vähe uuritud mistõttu on vaja täiendavaid katseid, et teha kindlaks, kas nende profiil kajastub vere EV-des.

Märksõnad: ekstratsellulaarsed vesiikulid; osteoartriit; miRNA; veri; sünoviaalvedelik

CERCS: B220 Geneetika, tsütogeneetika

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ABBREVIATIONS

ABs	apoptotic bodies
ACD	dextrose
AFSCs	amniotic fluid stem cells
AMPK	AMP-activated protein kinase
BMDCs	bone marrow-derived dendritic cells
BMSC	bone marrow stromal/stem cell derived extracellular vesicles
COL2A1	collagen type II alpha 1 chain
DEGs	differentially expressed genes
DGUC	iodixanol density gradient ultracentrifugation
DUG	differential ultracentrifugation
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
ESCRT	endosomal sorting complex required for transport
EVs	extracellular vesicles
exRNA	extracellular RNA
GPIb	glycoprotein Ib
HA	hyaluronic acid
HSNPs	hollow silica nanoparticles
IFNs	interferons
KPNA	karyopherin subunit alpha
MAPK	mitogen-activated protein kinase
MMP	matrix metallopeptidase
MSCs	mesenchymal stem cells
MVs	microvesicles

MVEs	multivesicular endosomes
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NGS	next generation sequencing
NTA	nanoparticle tracking analysis
OA	osteoarthritis
RA	rheumatoid arthritis
PBMCs	peripheral blood mononuclear cells
PG	prostglandin
PPAR	peroxisome proliferator-activated receptors
PSPMA	poly(3)-sulfopropyl methacrylate potassium
PTGS2	prostaglandin-endoperoxide synthase 2
RBCs	red blood cells
SDGUC	sucrose density gradient ultracentrifugation
SEC	size exclusion chromatography
SEVs	*Staphylococcus aureus* extracellular vesicles
SF	synovial fluid
TGB-β	transforming growth factor beta
TEM	transmission electron microscopy
TEx	tumour-derived exosomes
TLR	toll-like receptor
TMv	tumour-derived microvesicles
TSG101	tumour susceptibility gene 101

INTRODUCTION

Osteoarthritis (OA) is a disease that affects a significant part of the older population. Although there is no cure for OA, it is possible to manage the symptoms and if the symptoms are not manageable anymore, then endoprosthesis is done on severely damaged joints for patients with end-stage OA. For this reason, there is an interest in trying to find a biomarker for the disease through which it would be possible to diagnose OA in the early stages, and, through this, improve a patients' quality of life by slowing down the progression of OA. However, currently there are no good biomarkers to diagnose early OA prior to the appearance of radiological findings.

One possible way to detect OA biomarkers is by analysing the synovial fluid (SF) of OA patients, however, it is not the best diagnostic method. SF samples are obtained from patients through joint aspiration which is an invasive method and generally conducted only prior to joint arthroplastic surgery. The SF is not used for diagnostic purpose and for research, only perioperatives samples are often available. Therefore, even if the SF does contain possible biomarkers for the disease, it is not a preferred diagnostic method. On the other hand, obtaining and analysing patients' blood samples is much more efficient and plausible. A proposed method for OA diagnosis is through the analysis of extracellular vesicles (EVs) as both the SF and blood contain EVs.

EVs are vesicles that are involved in cell-to-cell communication and transport of different molecules. Many studies have already demonstrated their possible role in OA pathogenesis as well as their possible use in OA therapeutics as the function of these vesicles seems to depend on the cell they are derived from . EVs contain different molecules including RNA and are capable of safely transporting them due to their phospholipid bilayer that protects their cargo from degradation.

The general aim of this study is to give an overview of the current knowledge regarding the general transcriptomic profile of OA and, more specifically, the transcriptomic profile of SF and blood EVs. I hope to achieve this by first giving a general overview of the transcriptomic profile of OA and then introducing several OA studies regarding EVs derived from tissues, specifically SF and blood. I will analyse the role of the detected RNA in SF and plasma derived EVs and evaluate the potential of applying EVs as biomarkers to diagnose OA.

This study was carried out at the Institute of Bio- and Translational Medicine.

1. LITERATURE OVERVIEW

1.1. Blood components

Human blood is composed of 78% water and 22% solids. Red blood cells (erythrocytes), white blood cells (leukocytes) and platelets (thrombocytes) make up the solid part of the blood (Ameta *et al.*, 2017). Red blood cells are flexible and have an oval biconcave shape. Mature red blood cells have no nucleus and are missing most organelles to accommodate haemoglobin which makes up about 96% of red blood cells dry content (by weight), and 35% of total content including water. Haemoglobin transports oxygen from the lungs around the human body (C.-L. Wang *et al.*, 2017). White blood cells are very important in both the innate and adaptive immune system and there are approximately 4,000 to 10,000 white blood cells per microlitre of blood in an adult human body. They can be separated into four subclasses: eosinophils, lymphocytes, monocytes and neutrophils (Togacar *et al.*, 2019). Platelets are fragments of cytoplasm, derived from megakaryocytes that do not have a nucleus and have a lifespan of eight to ten days. They participate in different physiological responses such as homeostasis, thrombosis, wound healing and immunity. 150-450 billion platelets circulate in the human body per litre of blood and a turnover of 100 billion platelets occurs daily to maintain a physiological platelet concentration (Lefrançais and Looney, 2019). Figure 1 shows the ontogeny of the different types of blood cells mentioned above.

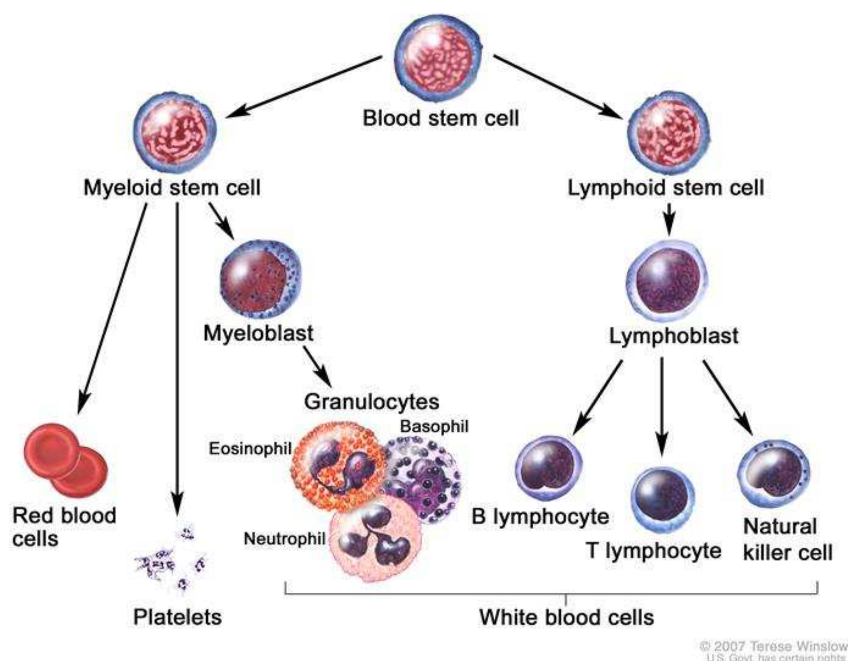

Figure 1. Distribution and origin of various blood components (*NCI Dictionary of Cancer Terms*, 2011).

Blood plasma is a protein-rich solution in which red and white blood cells as well as platelets are suspended (Leeman *et al.*, 2018). It is the pale yellow liquid fraction of blood that makes up approximately 55% of the body's total blood volume (C.-L. Wang *et al.*, 2017). Blood serum is differentiated from blood plasma by the absence of fibrinogens and clotting factors (Leeman *et al.*, 2018).

1.2. Synovial Fluid

Synovial joints, such as the wrist, knee, ankle, shoulder, and hip joints, are freely moveable joints that allow for a wide range of motions. In a healthy synovial joint (Figure 2), ends of the bones are covered with a smooth (hyaline) cartilage layer which functions as a shock absorber and forms a gliding surface (Tamer, 2013). Synovial joints are surrounded by the joint capsule, which has an external dense fibrous layer and an internal synovial membrane (synovium). The synovium contains macrophage-like synoviocytes (Type A) and fibroblast-like synoviocytes (Type B). Type A synoviocytes are located within the superficial layer of the synovium whereas type B synoviocytes lie in the deeper layer and secrete hyaluronic acid (HA) and collagens into the synovial fluid (SF) (Ando *et al.*, 2010). The SF is an ultra-filtrate of blood plasma filtrated through the synovial membrane and it functions as a biological lubricant while also containing nutrients and regulatory cytokines. Molecules that are said to play a role in lubrication of the joint, either alone or together, are proteoglycan 4, HA and surface-active phospholipids, all of which are secreted from synoviocytes. Within the joint, HA protects the cartilage and transports nutrients to the avascular cartilage (Tamer, 2013).

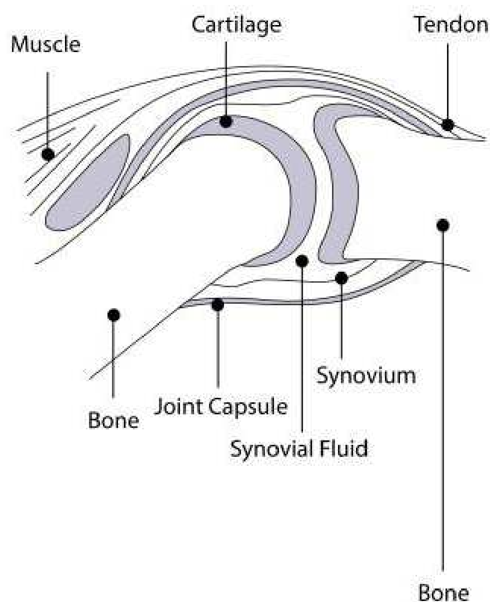

Figure 2. Structure and components of a healthy synovial joint. (Tamer, 2013)

1.3. Transcriptomics

Transcriptomics is the study of a cell's transcriptome which is all of the RNA transcripts found within a cell (Lowe *et al.*, 2017;Pertea, 2012). mRNA is a protein-coding RNA molecule while the rest of the RNA molecules present in a cell are non-coding. Ribosomal RNA (rRNA) and X-inactive specific transcript RNA (XIST RNA) are long non-coding RNA molecules (lncRNA) while microRNA (miRNA), small interfering RNA (siRNA), repeat associated small interfering RNA (rasiRNA), small nucleolar RNA (snoRNA), small nuclear RNA (snRNA), piwi-interacting RNA (piRNA), Y RNA and vault associated RNA (vtRNA), to name a few, are small non-coding RNAs (ncRNA). All these RNA molecules and their main functions are listed in Table 1.

Table 1. Summary of RNA molecules and their functions. (Dogini *et al.*, 2014; Gerstberger *et al.*, 2014)

RNA molecule	Function
messenger RNA (mRNA)	Contains the codons in a specific order from which proteins are synthesized.
transfer RNA (tRNA)	Transports amino acids to the ribosome to form a protein chain
ribosomal RNA (rRNA)	Protein synthesis
X-inactive specific transcript RNA (XIST RNA)	Inactivates the X chromosome
microRNAs (miRNA)	Help to regulate post-transcriptional gene expression
small interfering RNAs (siRNA)	Take part in gene regulation and help in the defence against virus and transposon activity
repeat associated small interfering RNAs (rasiRNA)	Take part in the formation of the centromere
small nucleolar RNAs (snoRNA)	Participate in the modification and processing of rRNA
small nuclear RNAs (snRNA)	Involved in the spliceosome complex
piwi-interacting RNAs (piRNA)	Regulate transposon activity
Y RNA	Acts as RNA chaperons, participates in DNA replication and in the immune response
vault associated RNAs (vtRNA)	Part of the vault ribonucleoprotein (RNP) complex and down regulates mRNA targets

1.4. Nature, origin and function of extracellular vesicles

Extracellular vesicles (EVs) have been found in archaea, bacteria and eukaryotes (Armstrong and Wildman, 2018) and they been found in all body fluids hinting towards their physiological importance (Dickhout and Koenen, 2018). They can be detected in blood plasma and serum, urine, saliva, amniotic fluid and breast milk (Kalra *et al.*, 2012) as well as synovial fluid (Miyaki and Lotz, 2018). EVs can also be found in semen, bronchial fluid, cerebral spinal fluid, tears, bile, gastric acid (Doyle and Wang, 2019), liver, brain, and placenta (Panteleev *et al.*, 2017). Hematopoietic cells such as B lymphocytes, dendritic cells, T lymphocytes and mast cells secrete EVs as well as non-hematopoietic cells such as intestinal epithelial cells, neuroglial cells and tumour cells. Platelets have also been shown to release EVs (Caby *et al.*, 2005).

EVs are membrane enclosed vesicles that used to be regarded as inert cellular debris released as a result of cell damage or dynamic plasma membrane turnover (Miyaki and Lotz, 2018). It has since been proven that EVs play an important role in cell-to-cell signalling in both normal physiological processes as well as pathological progression (Abels and Breakefield, 2016). EVs influence recipient cell function through the transferral of information. These signals can be transmitted by biomolecules such as proteins, lipids, nucleic acids and sugars *(Yáñez-Mó et al.*, 2015). EVs also have a phospholipid bilayer that protects the nucleic acids from degradation (H. Wang *et al.*, 2019).

Due to their mode of biogenesis, EVs have been divided into three subgroups: exosomes, ectosomes (also referred to as microvesicles), and apoptotic bodies (Kalra *et al.*, 2012) as described in Figure 3.

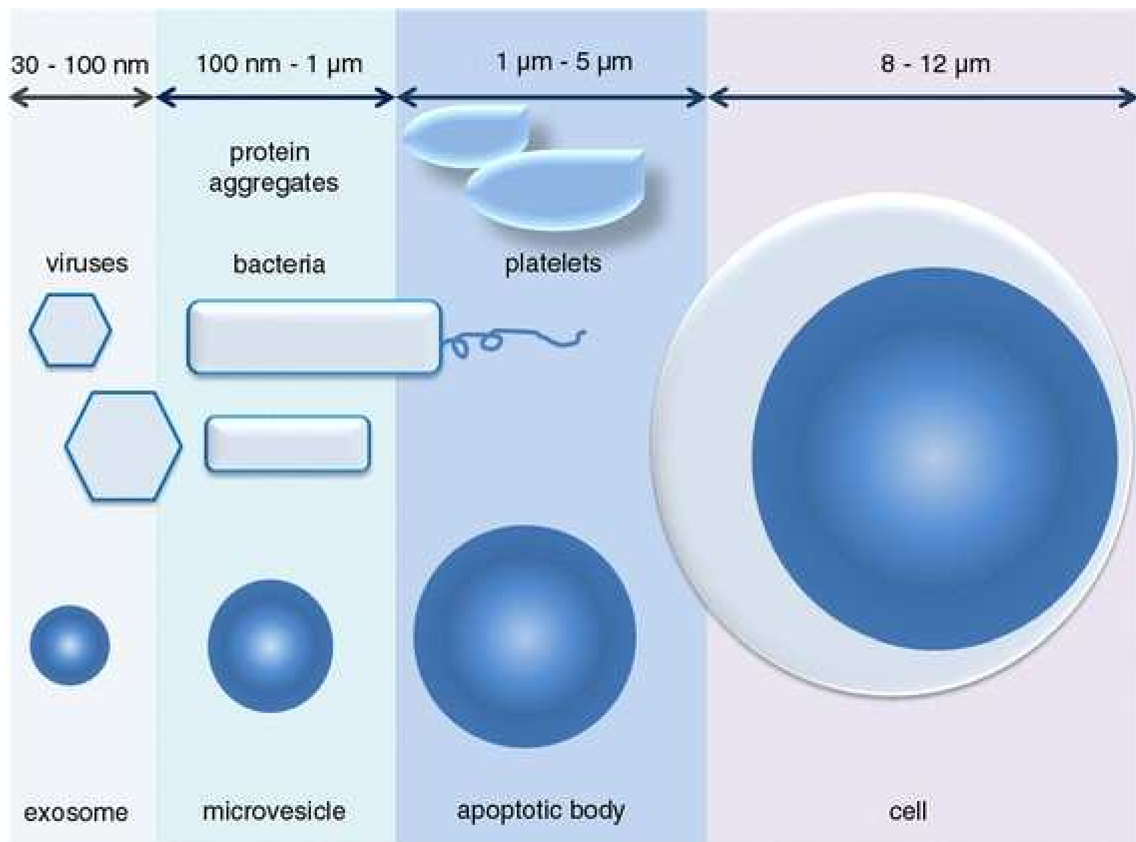

Figure 3. Rough size range of extracellular vesicles and a comparison to other molecules. (György *et al.*, 2011)

1.4.1. Exosomes

Exosomes are typically 30-100 nm in diameter (van Niel *et al.*, 2018). They are formed by the inward budding of the endosomal membrane during the maturation of multivesicular endosomes (MVEs) and are secreted once the MVEs fuse with the plasma membrane (van Niel *et al.*, 2018) as shown in Figure 4 (p.13). Endosomes are divided into three subtypes; early, late and recycling. Early endosomes that fuse with endocytic vesicles are destined for degradation, recycling or secretion. Remaining endosomes transform into late endosomes during which cytosolic bodies, nucleic acids, and lipids are sorted into small vesicles. Late endosomes containing these vesicles are named MVEs. MVEs that fuse with the lysosome are fated for degradation but if they fuse with the cellular membrane, then they are released as exosomes (Abels and Breakefield, 2016).

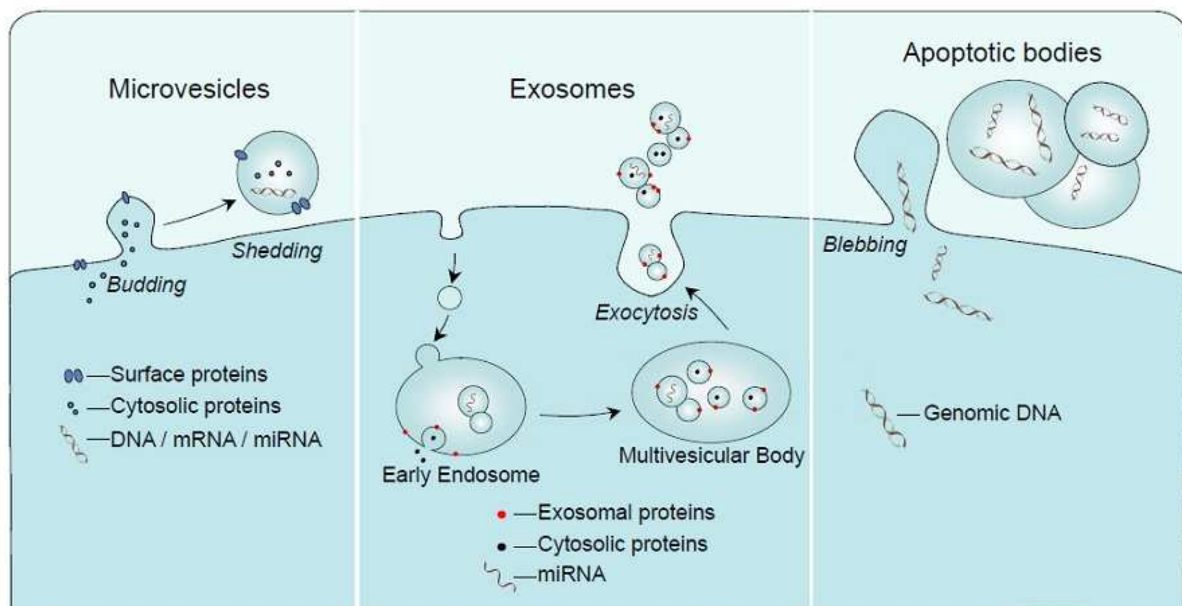

Figure 4. Different modes of biogenesis of microvesicles, exosomes, and apoptotic bodies (Lawson, 2017).

Exosomes released from parental cells have been shown to interact with target cells, influencing their behaviour and phenotype features. The genetic material of exosomes gets delivered via receptor-ligand interactions, direct fusion of membranes, or endocytosis (Y. Zhang *et al.*, 2019). During migration, the integrity of exosomal cargo is protected (Joo *et al.*, 2020) as demonstrated by RNA cargo remaining intact and resistant to RNase activity during migration (Valadi *et al.*, 2007). Exosomes are also capable of mediating intercellular communication throughout the body via the blood without inducing immune responses (Joo *et al.*, 2020). However, in the immune system this exosome mediated intercellular communication has been shown to influence antigen presentation, immune activation, immune suppression, and immune tolerance demonstrating the function of exosomes in immunoregulation (Y. Zhang *et al.*, 2019). Depending on their specific characteristics, exosome can be used in disease diagnosis, for drug delivery, and as therapeutic agents (Joo *et al.*, 2020).

1.4.2. Microvesicles

Microvesicles (MVs) are also known as shedding vesicles, ectosomes, shedding bodies, and microparticles (Tricarico *et al.*, 2016). The size of a normal vesicle ranges from 50 to 1000 nm but, in the case of oncosomes (MVs released from cancer cells), they can also be as big as 10 µm (van Niel *et al.*, 2018). MVs are shed from activated or apoptotic cells (Ridger *et al.*, 2017). When shed from normal cells, the rate of release is slow but MVs are released from

tumours at a constant rate (György *et al.*, 2011). The formation of MVs is caused by the redistribution of phospholipids and the contraction of the actin-myosin machinery. ADP-ribosylation factor 6 causes a cascade that activates phospholipase D. This leads to the phosphorylation of the extracellular signal-regulated kinase on the plasma membrane activating the myosin light chain kinase which is followed by the outward budding and fission of the plasma membrane resulting in the release of MVs into the extracellular space (Abels and Breakefield, 2016) as shown in Figure 4 (p.13).

MVs have been shown to have a variety of functions including playing a role in cell-to-cell communication (van Niel *et al.*, 2018) and having procoagulant activity (György *et al.*, 2011). As well as this, MVs have also been shown to cause a pro-inflammatory response when interacting with naïve endothelial cells (Ridger *et al.*, 2017), secreting IL-1β, contributing to the proinvasive nature of tumours, oncogenic cellular transformation and participating in feto-maternal communication (György *et al.*, 2011).

1.4.3. Apoptotic bodies

Apoptotic bodies (ABs) are also referred to as apoptotic cell-derived microparticles and apoptotic blebs. ABs are produced when the nuclear and cytoplasmic components are packed into membrane-bound vesicles during cell disassembly (Hauser *et al.*, 2017) and are released as blebs from cells undergoing apoptosis (György *et al.*, 2011) as shown in Figure 4 (p.13). They are about 1 - 5µm in diameter**,** roughly comparable to the size of platelets (Figure 3, p.12) but they can also be 50-500 nm in size (György *et al.*, 2011). The presence of phosphatidylserine on the surface membrane as well as the presence of intact organelles within the vesicle are key characteristics of ABs (Akers *et al.*, 2013). They also contain intact chromatin and small amounts of glycosylated proteins (Doyle and Wang, 2019). ABs may also include mRNAs, lncRNAs, rRNA, miRNAs, or fragments of intact RNA molecules (Zhu *et al.*, 2017).

The composition of apoptotic bodies varies; some are made of condensed nuclear chromatin while others are made of cytoplasmic components. This affects their physical properties which can affect how ABs are purified when the methods use density, gravity, or charge to separate them. This difference is due to tightly packed DNA being denser and it carries a negative charge. In addition to this, they are stained by different markers which is a result of ABs containing either DNA or cytoplasmic proteins. This led to ABs being divided into nuclear apoptotic bodies and cytoplasmic apoptotic bodies (Hauser *et al.*, 2017).

ABs have been shown to take part in the horizontal transfer of oncogenes and DNA, yielding presentation of T cell epitopes upon uptake by phagocytic cells and representation of B cell autoantigens. Uptake of ABs has been shown to lead to immunosuppression (György *et al.*, 2011). Signals in apoptotic bodies attract phagocytes to apoptotic sites for apoptotic cell clearance (Hauser *et al.*, 2017). Instead of isolating ABs, most studies use co-cultures of cells that are going through apoptosis to investigate their functions (György *et al.*, 2011).

1.4.4. Proteins, lipids and nucleic acids in EVs

EV cargo reflects the intracellular origin of the cargo as well as they type of cell from which the vesicle was derived (Sedgwick and D'Souza-Schorey, 2018). The most commonly found proteins in EVs are those that also participate in their biogenesis – this includes the proteins that are associated with the endosomal pathway (Abels and Breakefield, 2016). There have been reports of transcription factors contained in EVs (Kalra *et al.*, 2012); however proteins associated with the endoplasmic reticulum, Golgi, and nucleus have not been found in smaller EVs such as exosomes and MVs (Abels and Breakefield, 2016). Exosomes are enriched in major histocompatibility complex class II molecules (MHCII) and tetraspanins CD37, CD53, CD63, CD81, and CD82. The endosomal sorting complex required for transport (ESCRT) is a group of proteins that are necessary in the formation of MVEs. This pathway also needs additional proteins such as Alix and tumour susceptibility gene 101 (TSG101). MVs, on the other hand, tend to be enriched in integrins, glycoprotein Ib (GPIb), and P-selectin. They also carry more proteins with posttranslational modifications like glycoproteins or phosphoproteins. Lastly, ABs contain DNA-binding histones and are depleted in glycoproteins. Proteins such as MHC II, tetraspanins, ESCRT proteins, Alix, TSG101, and heat-shock chaperone proteins can be used as general EV biomarkers (Zaborowski *et al.*, 2015).

EVs are mainly enriched with small RNAs but DNA has also been found. Many of these RNAs are derived from ribosomal 18S and 28S rRNAs and tRNAs. RNA molecules found includes mRNAs, miRNAs, tRNAs, long and short non-coding RNAs, tRNA fragments, piRNAs, vtRNAs, and Y RNAs. Most of these molecules are 200 nucleotides long and most are also fragmented. Circular RNAs are also enriched and stable in EVs (Abels and Breakefield, 2016). About 15% of EV-RNA consists of miRNA and tRNAs and the amount of RNA molecules can vary as oncosomes contain more RNA molecules than EVs from normal cells (Zaborowski *et al.*, 2015).

DNA transported by EVs is referred to as EV-DNA and its size varies from 100 bp to 2.5 kB. Long double stranded DNA fragments can also be found in EV pellets, but they are unprotected from DNase activity as they are not enclosed in the EV membrane. The functional significance of this cargo is unknown (Zaborowski *et al.*, 2015).

1.5. EVs in blood and synovial fluid

1.5.1. EVs in blood

Originally, EVs in blood plasma were named platelet dust and described as "subcellular materials originating from platelets in plasma and serum" (van Niel *et al.*, 2018). Majority of the EVs that circulate in plasma are of platelet origin, but their mode of biogenesis is still unclear. EVs have also been found to originate from both erythrocytes and leukocytes as well (Panteleev *et al.*, 2017).

In addition to EVs, blood also contains soluble proteins and lipoproteins as a result of which blood plasma and serum have high viscosity and density. Due to this, it is hard to accurately detect, isolate and evaluate the concentration of EVs (Clayton *et al.*, 2019), however, in spite of this, it has been calculated that there are approximately $5*10^9$ EVs per mL of plasma in a healthy person's blood (H. Wang *et al.*, 2019). EVs isolated from the serum of healthy humans have been reported to activate endothelial cells as well as having proangiogenic functions *in vitro* and *in vivo* (Takov *et al.*, 2018).

EVs partake in coagulation and inflammation in blood while higher EV levels have been associated with cardiovascular diseases, cancer, sepsis, and autoimmune diseases (Arraud *et al.*, 2014). As well as this, it has been shown that the number of EVs originating from erythrocytes increases in the case of sickle-cell anaemia, beta-thalassemia, and other diseases but normally, there are less EVs of erythrocytes origin than of platelet or leukocyte origin (Panteleev *et al.*, 2017).

1.5.2. EVs in synovial fluid

EVs in SF have been shown to have pro-inflammatory properties (Foers *et al.*, 2018) and to facilitate coagulation (Berckmans *et al.*, 2002). As well as this, SF-EVs have been shown to play a role in the progression of OA (Domenis *et al.*, 2017).

Domenis *et al.* investigated the immune regulatory properties of OA SF-EVs on pro-inflammatory M1 macrophages differentiated from peripheral blood mononuclear cells (PBMCs). Treatment of M1 macrophages with SF-derived exosomes resulted in the release of IL-1β and induced the released of chemokines CCL8, CCL15, CCL20, and CXCL1 while

downregulating the production of CCL7. There was also a release of MMP12 and MMP7 while MMP8 production was inhibited (Domenis *et al.*, 2017). IL-1β is one of the main proinflammatory cytokines involved in OA pathophysiology (Kapoor *et al.*, 2011) while CCL8 and CCL7 are chemoattractants that recruit monocytes to sites of trauma and infection and, in the case of OA, to sites of inflammation (Yung and Farber, 2013). CCL20 induces changes in phenotype and catabolic gene expression in chondrocytes (Alaaeddine *et al.*, 2015) while CXCL1 induces chondrocyte hypertrophy and apoptosis during cartilage development as well as playing a role in inflammation (Wenke *et al.*, 2011). Increased MMP12 expression has been shown to be expressed in the cartilage and subchondral bone of OA patients but it was absent in normal controls (Kaspiris *et al.*, 2015). MMP7 degrades various ECM components such as cartilage proteoglycans and it is overexpressed in OA (Y. Tao *et al.,* 2015). MMP8 causes hyperinfiltration of joints with neutrophils as it downregulates neutrophil apoptosis and clearance (Rose and Kooyman, 2016).

1.5.3. Transcriptome of EVs in blood and SF

More detailed studies of EVs in blood have been performed but an in-depth study of the OA-EV transcriptomic profile is yet to be performed. Amorim *et al.* performed a whole transcriptomic analysis of EVs isolated from the plasma of 5 healthy women. They concluded that 73.25% of the reads were short non-coding RNAs, 24.46% were protein coding RNAs, 2.16% long non-coding RNAs and 0.1% pseudogenes. In the short non-coding RNA category, 57.29% were tRNAs, 14.83% were mitochondrial rRNAs, 13.23% were miscellaneous RNAs and 12.86% were miRNAs. Amongst the miscellaneous RNA category were Y RNAs (57.45%), SRP_7SL_RNAs (39.44%), vtRNAs (2.99%) and 7SK_RNAs (0.11%) (Amorim *et al.*, 2017).

Hunter *et al.* performed a more in-depth transcriptomic analysis of human plasma and PBMCs by focusing on the miRNA content within MVs. Results showed that 104 miRNAs in MVs and 75 miRNAs in PBMC samples were significantly expressed while 71 of these were co-expressed among each sample. miR-223, -484, -191, -146a, -16, -26a, -222, -24, -126, and -32 were detected in plasma microvesicles, while miR-222, -24, -126, and -32 were the top expressed. miR-150, -146b, -19b and -20a were among the highest miRNAs detected in PBMC, but not in plasma microvesicles. However, both samples contained miR-223, -484, -191, -146a, -16, and -26a. miR-486 was the highest differentially expressed miRNA in the plasma MVs compared to PBMC. As many plasma MVs were platelet-derived, the miRNA expression of platelets was observed. There were 53 co-expressed miRNAs but there were

also many miRNAs unique to plasma MVs. miR-223 was the highest expressed miRNA in platelets but there was no observed expression of miR-484 in platelets. (M. P. Hunter *et al.*, 2008).

While the general transcriptomic profile of EVs in blood has been investigated, the same cannot be said for the general transcriptomic profile of EVs in SF regarding OA. However, some have investigated the SF-EV miRNA profile in the case of diseases such as OA. Kolhe *et al.* discovered that the SF-EV profile could be gender specific as the only miRNA significantly expressed by both genders was miR-504. Overall, they found that 69 miRNAs were significantly down-regulated, and 45 miRNAs were up-regulated in males while in females, 91 miRNAs were down-regulated and 53 miRNAs up-regulated (Kolhe *et al.*, 2017). Furthermore, Griswold *et al.* analysed the transcriptomic profile of SF extracellular RNA (exRNA) following knee trauma. They observed that the expression of 69 protein coding genes (44 up-regulated, 25 down-regulated) and eight miRNAs (3 up-regulated, five down-regulated) showed significant differences, especially MMP3, MMP21, MMP1 and miR-320a (Griswold *et al.*, 2018).

However, comparing the transcriptomic profile of the SF and blood in this case is quite difficult. Mainly because the blood is not taken from OA patients but rather from healthy patients. Secondly, one study focuses on blood EVs, another on blood MVs and lastly, SF exosomes. As these studies are not based on similar EV subtypes, comparing them is not accurate and therefore not possible.

1.6. Potential medical applications of EVs

EVs present a unique opportunity for the development of a new class of therapeutics due to their wide range of biological functions and capability to shuttle large molecules between cells (Wiklander *et al.*, 2019). Different types of EV molecules are being studied for numerous therapeutic applications. This includes the use of EVs in anti-tumour therapy, pathogen vaccination, immune-modulatory and regenerative therapies as well as drug delivery (Lener *et al.*, 2015).

1.6.1. EVs in anti-tumour therapy

Tumour-derived EVs have tumour-specific integrins which directs them towards the tumour or metastatic sites. Thus tumour-derived exosomes (TEx) and MVs (TMv) are ideal as carriers for therapeutic agents. The tumour microenvironment can be regulated by the delivery of inflammatory cytokines and by the elimination of suppressing factors. Rossowska *et al.*

focused on transforming growth factor beta (TGF-β1) and IL-12 (Rossowska *et al.*, 2019). TGF-β1 is an anti-inflammatory cytokine associated with tumour progression and metastasis (Principe *et al.*, 2014) while IL-12 is a proinflammatory cytokine that has high antitumour potential as well as high toxicity when applied in its recombinant form (Lasek *et al.*, 2014). Rossowksa *et al.* developed and analysed TGF-β1-deprived EVs with IL-12 cargo and the results showed that TEx from unmodified MC38 cells accelerated tumour growth while modified TEx, especially those deprived of TGF-β1, caused tumour growth inhibition (Rossowska *et al.*, 2019).

1.6.2. EVs in pathogen vaccination

EVs derived from gram-negative bacteria contains pathogenic components or virulence factors due to which they can be used as vaccination agents by inducing both innate and adaptive immunity. When bone marrow-derived dendritic cells (BMDCs) were treated with *Staphylococcus aureus* EVs (SEVs), they were discovered to have the vaccine adjuvant ability to induce adaptive immunity. The expression of co-stimulatory molecules was enhanced in BMDCs as were the production of pro-inflammatory mediators. The SEV vaccination was tested against *S. aureus*-induced pneumonia in a mouse model. $CD4^+$ T cells were transferred from SEV-immunized mice to naïve mice of which 70% showed an effective protective response to the infection, demonstrating that the vaccination induced by SEV-immunization is mediated mainly by $CD4^+$ cell responses (Choi *et al.*, 2015).

1.6.3. EVs in immunomodulatory therapies

Mesenchymal stem cells (MSCs) are important in maintaining tissue homeostasis (Xiuxiu Yin *et al.*, 2019) and have been isolated from bone marrow, adipose tissue, the skin, lungs and the umbilical cord (Kim *et al.*, 2019). By releasing inflammatory cytokines into the tissue microenvironment (Trapani *et al.*, 2016), MSCs have also been shown to participate in immunomodulatory effects in both acute and chronic diseases (Tan *et al.*, 2019). It has been suggested that the immunomodulatory effect of MSCs comes from the secreted EVs rather than from the MSCs themselves. Depending on the cellular origin and cytokine cargo of EVs, they can either have an immune-stimulatory or immune-suppressive effects (Kordelas *et al.*, 2019). EVs partake in tissue repair and immune regulation due to their ability to mediate MSCs paracrine mechanism (Trapani *et al.*, 2016).

1.6.4. EVs in regenerative therapies

Amniotic fluid stem cells (AFSCs) have already been shown to have beneficial effects in organ and tissue regeneration. These regenerative studies have been carried out on the kidney, heart, intestine, lung, bone, bladder, and muscle. AFSCs beneficial effects have been attributed partially to EVs which mediate paracrine intercellular communication. Antounians *et al.* demonstrated the regenerative capabilities of AFSC-EVs by treating nitrofen damaged lung epithelium cells with AFSC-EVs resulting in a significant reduction in the rate of cell death. They also found that the effect of AFSC-EVs on lung epithelial cell survival depends on the number of EVs administered (Antounians *et al.*, 2019).

As well as AFSC-EVs, there has also been studies to observe the regenerative potential of bone marrow stromal/stem cell derived EVs (BMSC-EVs). Qin *et al.* demonstrated the *in vitro* osteogenic potential of BMSC-EVs and furthermore, studied their capabilities to stimulate bone regeneration *in vivo.* In this study, the EV-group demonstrated accelerated bone regeneration and enhancement in repair. The area density and amount of newly formed bones was significantly increased in the EV-group when compared to the control quite clearly demonstrating the regenerative capabilities of BMSC-EVs (Qin *et al.*, 2016).

1.6.5. EVs in drug delivery

RNA based therapies have proven useful for their specificity and flexibility when it comes to targeting the diseased human genome, but common vehicles used for RNA drug delivery are usually immunogenic and/or cytotoxic. EVs, on the other hand, have proven to be proficient in drug delivery as they are biocompatible with mammalian cells. Due to this, they are capable of overcoming cellular and drug delivery barriers such as phagocytosis, immunogenicity and cytotoxicity (Usman *et al.*, 2018).

Usman *et al.,* studied the capabilities of EVs produced by human red blood cells (RBCs) for the delivery of therapeutic RNAs. These RBC-EVs were taken up by leukaemia cells and after 24 h of incubation, a clear uptake of Haemoglobin A was observed which had been absent in the untreated cells. Moreover, after a 24 h incubation period, ~99% of these cells were positive for fluorescence-labelled EVs demonstrating the capabilities of EVs in RNA drug therapies (Usman *et al.*, 2018).

1.7. Isolation of EVs

1.7.1. Differential ultracentrifugation

Differential centrifugation is the most commonly used method of isolating EVs, particularly exosomes, and it is based on several centrifugations, each of which increases in both speed and time resulting in smaller particle pellets. These centrifugations are carried out on the supernatant of the prior ones while the pellets are discarded with the aim of pelleting the exosomes (Cvjetkovic *et al.*, 2014). Floating cells are pelleted first (300 *g*, 10 ming), then ABs (2000 *g*, 20 min), MVs (16,000 *g*, 40 min), and then finally the exosomes (100,000 *g*, 1h) (Lee *et al.*, 2016).

This method is low cost, there is a reduced risk of contamination, allows for a large sample capacity, and it yields large amounts of EVs. However, it is a time consuming method, requires a large starting sample, the exosome preparation may be contaminated with MVs or protein aggregates and it is not suitable for use in a hospital setting (Lucchetti *et al.*, 2019). As well as this, there can be inconsistencies in isolated materials in different protocols due to altered centrifugation duration, relative centrifugal force, temperature and the type of rotor used (Cvjetkovic *et al.*, 2014) as a result of which it is hard to obtain a standardised isolation method.

1.7.2. Density gradient centrifugation

Density gradient centrifugation is a method that separates particles based on their density using sucrose or iodixanol (Onódi *et al.*, 2018). This method also allows for the separation of subcellular components like mitochondria, peroxisomes, and endosomes. There is also the possibility to obtain a pure preparation with no viral contamination when using iodixanol preparations, and no additional chemicals have to be used for this method. However, this is a complex method of isolation that could lead to the loss of sample or, in the case of sucrose density gradient preparation, contamination with viral particles (Konoshenko *et al.*, 2018).

1.7.3. Size exclusion chromatography

Size exclusion chromatography (SEC) is based on molecule separation due to size difference. A solution containing EVs is run through a column containing sepharose. The sepharose beads have pores and any particles larger than 75 nm, such as EVs, cannot enter the beads and they elute earlier than the particles that are delayed due to the increased path length. In comparison to differential centrifugation and density-gradient ultracentrifugation, protein complexes and vesicle aggregation does not occur when using SEC (Böing *et al.*, 2014).

1.7.4. EV isolation from SF

EV isolation from SF has proven to be a challenge due to both the viscosity and composition of the fluid (Foers *et al.*, 2018). To decrease the viscosity of SF, it is treated with hyaluronidase which has been shown to be a beneficial step in the isolation of CD44+ EVs (Boere *et al.*, 2016). The main method of isolation has been differential ultracentrifugation where EVs have been isolated using ultracentrifugation with or without further sucrose density gradient purification. These preparations have been shown to include considerable levels of non-EV material (Foers *et al.*, 2018).

Foers *et al.* used differential ultracentrifugation (DUG) to prepare the EVs and used Western blot for analysis and while EV markers were detected, there was also serum albumin, high density lipoproteins (ApoA-I marker) and other contaminants that are not associated with EVs. When analysed with transmission electron microscopy, amorphous material and areas of dense aggregation were observed. Sucrose density gradient ultracentrifugation (SDGUC) was also shown to not deplete high density lipoproteins from EV isolations (Foers *et al.*, 2018)

EVs have also been isolated from SF by SEC. When the achieved fractions were analysed by Western blot, fractions 2-4 were shown to include EV markers, serum albumin was eluted in fractions 5 and 6, and ApoA-I was present in fractions 4-8. Fibronectin was detected in fractions 2-6 and was abundant in fractions 3-5. Negligible levels of serum albumin and ApoA-I were detected in fractions 2 and 3 (Foers *et al.*, 2018).

These results show that SEC is a more efficient method of EV isolation from SF than DUG and SDGUC. It was more effective in separating them from serum albumin and high-density lipoproteins which remain more abundant when EVs were isolated from SF through differential centrifugation and sucrose density gradient ultracentrifugation.

1.7.5. EV isolation from blood

EVs are usually isolated from blood plasma instead of blood serum as there is a higher yield of EVs and >50% of serum EVs may be platelet derived. The use of an anticoagulant in blood samples results in calcium chelations as well as the inhibition of both protease activity and platelet activation. Thus, when choosing an anticoagulant, it is important to take into consideration further applications. The use of heparin-based anticoagulants is discouraged as it is associated with false-negative PCR reads. This is thought to happen because heparin

competes with primers and/or enzymes in binding to nucleic acids. As well as this, heparin can bind EVs, block EV uptake by cells, and lower the platelet activation threshold. Blood treated with ethylenediaminetetraacetic acid (EDTA), dextrose (ACD), or citrate resulted in the apparent count of EVs being lowered as calcium chelators promote rapid association of EV with platelets (Witwer *et al.*, 2013). It has been shown that platelet EV counts were lower in blood samples collected in citrate or EDTA than in those collected in protease inhibitors (Szatanek *et al.*, 2015)

Ultracentrifuge based EV enrichments for plasma have shown high levels of contaminants such as high-density lipoproteins and serum albumin (Foers *et al.*, 2018). Isolation of EVs by differential centrifugation has also proven to be ineffective due to the fact that the viscosity of the plasma affects the recovery of vesicles (Böing *et al.*, 2014). Thus, combining different methods of isolation and purification is best when isolating EVs from blood.

SEC has proven to be efficient in EV purification from complex biological fluids such as blood and blood plasma. It avoids high centrifugal force which usually leads to vesicle rupture and aggregation and it is also capable of separating EVs from serum albumin (Foers *et al.*, 2018). Despite this, Karimi *et al.* showed that SEC alone is not effective in removing lipoproteins and plasma proteins from fractions that also contained EVs and EV markers. SEC removes lipoprotein particles that have a similar density to EVs but are different sizes. The solution to this was combining density gradient centrifugation with SEC as density gradient is capable of separating EVs from lipoproteins of similar size but have different densities. This resulted in Karimi *et al.* obtaining a purer sample as the lipoprotein particles and plasma particles were removed. To further increase the yield of EVs, the plasma was ultracentrifuged and the pellets were dissolved in PBS. This has the added benefit of being able to start with any volume of plasma. Then this mixture was purified using density gradient, and SEC. As a result, more vesicles were present in certain fractions when compared to fractions where ultracentrifugation were not used (Karimi *et al.*, 2018).

Onódi *et al.* isolated EVs from blood plasma by iodixanol density gradient ultracentrifugation (DGUC) followed by bind-elute SEC. The use of DGUC decreased the lipoprotein contamination while also keeping the high separation efficiency. Further purification through bind-elute SEC resulted in a decreased yield but lipoproteins and albumin were removed while certain blood components were not (Onódi *et al.*, 2018).

Serum is a rich source of EVs but separating EVs from serum proteins and non-EV lipid particles has proved to be a challenge. Exoquick plus (polymer-based precipitation; System

Biosciences) and SEC fraction 7 and 8 yielded the largest number of particles isolated but lipoprotein markers such as APOB and APOE were most abundant using these methods. However, if SEC fractions were purified using iodixanol density gradient centrifugation followed by ultracentrifugation, a lower particle to protein ratio was observed (Brennan *et al.*, 2020).

In conclusion, there seems to be no one certain method that can be used to isolate EVs from blood. Each method seems to have their own downfalls which can be compensated for by combining them with other isolation and purification methods.

1.8. Analysis of EVs

1.8.1. Nanoparticle tracking analysis

Nanoparticle tracking analysis (NTA) is based on the "characteristic movement of (nano)particles in solution according to the Brownian motion" (Bachurski *et al.*, 2019). Brownian motion is used to describe the uncontrolled movement of particles in a liquid as they collide with other particles (Floyd *et al.*, 2017). The movement of these particles is recorded by a camera when light is scattered by a laser illuminating particles during which the smaller particles move faster than the bigger ones. Through the use of NTA, it is possible to determine both the size and concentration of samples (Bachurski *et al.*, 2019).

The NTA devices used are NanoSight NS300 (Malvern, UK) and ZetaView (Particle Metrix, Germany). Both devices allow for the determination of size and concentration of samples but differ in their composition of hardware and software (Bachurski *et al.*, 2019).

Both devices fail to detect EVs smaller than 50 nm. ZetaView is more accurate in measuring concentration while NS300 is more accurate in measuring size. ZetaView is also more capable of accurately measuring sequentially diluted EV samples and has also been proven to be more sensitive to the changes in concentration of an EV sample after multiple freeze-thaws which results in EV degradation. NanoSight NS300, on the other hand, does not detect these differences (Bachurski *et al.*, 2019).

1.8.2. Other methods of analysis

Other methods used in the analysis of EVs are western blot and transmission electron microscopy (TEM). Tetraspanins CD9, CD63 and CD81 are general EV markers (Campos-Silva *et al.*, 2019) used in these methods to ascertain the presence of these vesicles. Western blotting also allows to attain the purity of the sample (Mahmood and Yang, 2012).

TEM can be used to determine the quality and purity of EVs (Rikkert *et al.*, 2019) but it can also be used to attain information on the biochemical properties of EV surface proteins with the aid of immunogold labelling. EVs have to be fixed and dehydrated when using TEM (Cizmar and Yuana, 2017) and the method itself is both time consuming and technically challenging (Bachurski *et al.*, 2019). However, it is possible to analyse frozen samples through the use of cryo-TEM without having to fixate/dehydrate the EVs and it is possible to detect EV surface proteins using immunogold labelling but this method can also be technically demanding (Cizmar and Yuana, 2017).

1.9. Osteoarthritis

Osteoarthritis is the most common form of arthritis and it affects the tissues of the joint such as the cartilage, bone, and capsule, but also ligaments, and muscles. Typically, this disease affects the hips, hands, and knees but it can also develop in other joints. It is considered a chronic disease and has no preventable cure. However, there are treatments that can reduce pain, improve function, and delay the progression of the disease (*OARSI*, 2016). OA can be defined in three ways: clinically, radiologically, or symptomatically. Clinical OA is diagnosed based on symptoms and a physical examination, radiological OA is diagnosed based on the presence of osteophytes, and symptomatic OA is diagnosed based on a patient experiencing frequent pain and radiographic evidence (Lawrence *et al.*, 2008).

The prevalence of OA has been increasing throughout the years. It has been estimated that 27 million US adults (Lawrence *et al.*, 2008) and >40 million Europeans have OA (Kingsbury *et al.*, 2014). In the UK, approximately 8.5 million have clinical OA. The prevalence of which increases with age; 13.9% of people aged 25 and over had OA of at least one joint, while 33.6% of people aged 65 and over had OA (Neogi, 2013). In Estonia in 2016, it was estimated that there were 33,018 men and women of all ages with OA, the prevalence of which increased significantly after the age of 35. After the age of 45, the number of women with OA doubled relative to men (*Tervise Arengu Instituut*, 2016).

A characteristic of OA is unpredictable, intermittent pain which has been shown to have a bigger impact on the quality of life than constant pain in OA patients. The pain negatively impacts an individual’s mood, participation in social and recreational activities, and sleep (Neogi, 2013). It also impacts an individual’s capability to do everyday activities which, as a result, affect the quality of life. Many with OA avoid exercise/sport, gardening, and climbing stairs while also requiring assistance with cleaning and dressing (D. J. Hunter and Riordan, 2014).

1.9.1. OA pathogenesis

OA has many risk factors including obesity, sex, race and ethnicity, genetics, nutrition, smoking, and injuries/trauma to the joint. In the case of genetic predisposition, there is a possibility that it will not develop unless if there has been joint trauma or if they express one or more of the other risk factors (Kapoor, 2015). OA pathogenesis is characterised by the degradation of the articular cartilage, remodelling of the subchondral bone, formation of osteophytes, synovitis, degeneration of menisci, and hypertrophy of the joint capsule (Robinson *et al.*, 2016). Figure 5 describes characteristic differences between a normal and OA joint.

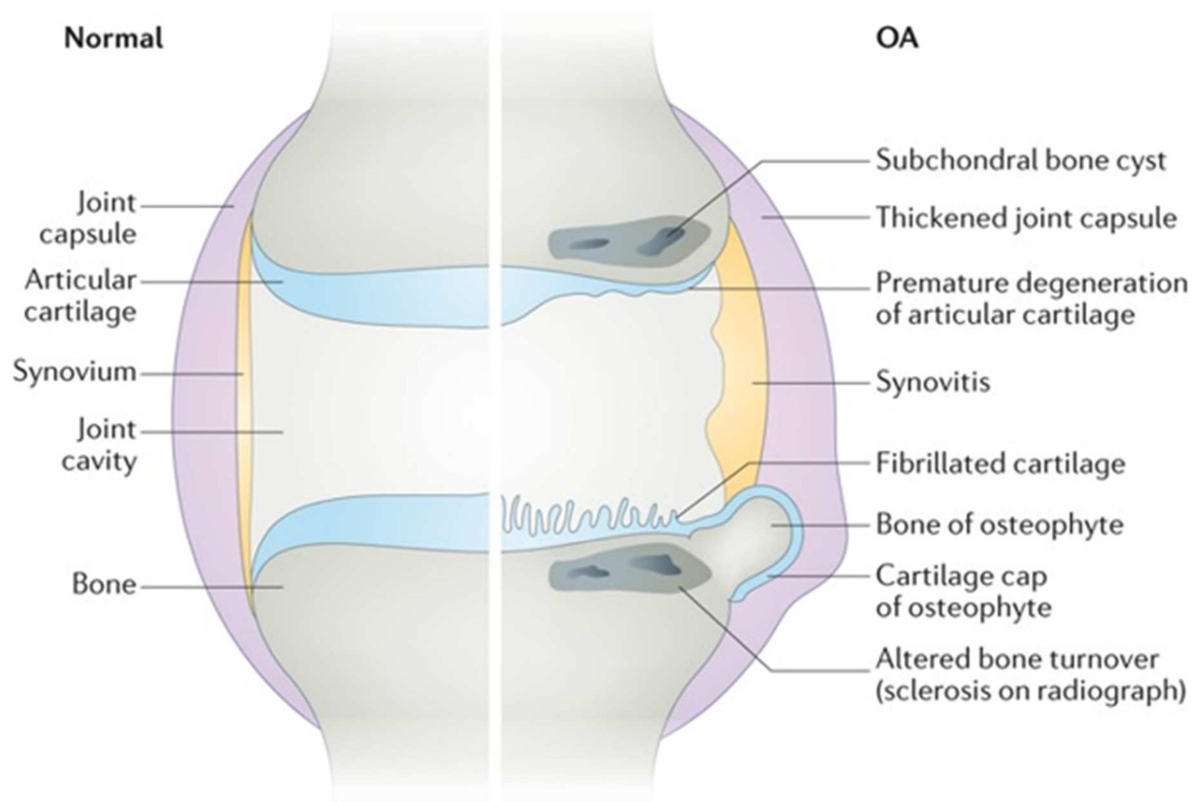

Figure 5. A comparison between a healthy and OA joint. The changes that occur in the progression of the disease are highlighted (Robinson *et al.*, 2016).

The articular cartilage is composed of the extracellular matrix (ECM) and chondrocytes (Fox *et al.*, 2009) and contains no blood vessels, nerves, or lymphatic vessels (Blalock *et al.*, 2015). The ECM itself is made up of water, type II collagen fibres, and proteoglycans, and to a lesser extent, glycoproteins and non-collagen proteins. All of these components facilitate water retention within the ECM, which is important for maintaining the mechanical properties of the

articular cartilage, like its elasticity and tensile strength (Fox *et al.*, 2009;Heijink *et al.*, 2012). Proteoglycans have a rapid turnover rate while collagen does not. The turnover of proteoglycans and collagens is mediated by chondrocytes, which synthesize these along with the proteolytic enzymes needed for their breakdown. Chondrocytes themselves are influenced by both growth factors and cytokines (Man and Mologhianu, 2014). Osteoarthritis occurs when the chondrocytes are incapable of maintaining the homeostasis between the synthesis and degradation of the ECM components. When this homeostasis is disrupted, the water content in the ECM increase while the proteoglycan content decreases which weakens the collagen network as there is a decrease in the synthesis of type II collagen and an increase in the breakdown of existing collagen. As well as this, apoptosis of chondrocytes increases due to compensatory mechanisms. At first, the increased synthesis of matrix molecules and proliferation of chondrocytes is capable of maintaining the integrity of the cartilage, but eventually the loss of chondrocytes and changes in the ECM cause the development of osteoarthritis (Heijink *et al.*, 2012).

The subchondral bone is separated from the articular cartilage by a zone of calcified cartilage and it consists of the subchondral bone plate, trabecular bone and bone marrow space. The properties of the subchondral bone are modified through remodelling and modelling. Remodelling is a result of bone resorption and new bone formation of a resorbed surface. Modelling, on the other hand, causes changes in the architecture and volume of the bone. As a result of osteoarthritis, these mechanisms can be altered causing structural changes in the subchondral bone. These changes result in an increased thickness of the subchondral bone plate, modifications in the architecture of subchondral trabecular bones and the formation of osteophytes which is the formation of new bone at the joint margins (Man and Mologhianu, 2014).

The joint capsule is very important in joint functionality as it seals the SF within the joint, limits joint movement resulting in passive stability and provides active stability as well. Collagen fibres within the bone adhere to bone using fibrocartilaginous attachment. Blood vessels and nerves pass through the capsule to supply it and the synovium (Blalock *et al.*, 2015).

The synovial membrane consists of metabolically active cells named synoviocytes, which are important as they nourish chondrocytes and remove metabolites and products of matrix degradation. Synovitis occurs due to the inflammation of the synovium and it is responsible for many clinical symptoms (Sellam and Berenbaum, 2010). Synovitis is thought to be caused by cartilage matrix proteolytic degradation products and other factors such as microcrystals

and abnormal mechanical stress. These components are released into the SF and go through phagocytosis because of the macrophages in the synovial lining, which results in the synthesis of mediators that lead to the inflammation of the synovium. These mediators diffuse into the cartilage through the SF and this continues in a vicious circle which results in increased cartilage degradation and an increase in inflammation. Synoviocytes are also capable of producing proinflammatory cytokines such as IL-1β, IL-6 and TNFα which are thought to mediate the progression and pain related to this disease (Man and Mologhianu, 2014).

Another feature of OA are meniscal tears. Menisci are fibrocartilaginous, wedge-shaped structures that take part in joint stability, load distribution, shock absorption, and lubrication (Blalock *et al.*, 2015). Degraded menisci appear torn, fissured, fragmented, macerated or completely destroyed and this process begins within the substance of the tissue (Man and Mologhianu, 2014). Loss of a substantial amount of meniscal tissue permanently changes the biochemical and biological environment of the knee joint (Heijink *et al.*, 2012). Tissue disruption occurs at the inner rim and spreads to the articular surfaces of the meniscus. This leads to a loss of meniscus tissue in the avascular zone. Type I and type II collagen content decreases from the surface, middle and deep zone while the proteoglycan content increases in the menisci. All of this contributes to meniscal degeneration and reduced tensile strength. The ability of the meniscus to withstand loading and force transmission results in degenerative tears (Man and Mologhianu, 2014).

1.9.2. Current management strategies

Clinical end stage treatment of OA is artificial joint replacement but treating early stage OA through non-surgical techniques is preferable for patients (T. Sun *et al.*, 2018). A novel strategy for OA treatment is synergistic therapy (Ji and Zhang, 2019). However, in general, OA is treated with the help of medications, nondrug therapies or a combination of both (Arthritis Foundation, n.d.).

Synergistic therapy combines enhanced joint lubrication and drug delivery in OA treatment (Ji and Zhang, 2019). For instance, nanoparticles were used for both joint lubrication and as nanocarriers for aspirin. The results showed that 67% of aspirin was released in 24 hrs, 74.5% in 72 hrs while without a carrier, 100% was released in 5 hrs (G. Liu *et al.*, 2014).

Numerous different pain and anti-inflammatory medicines exist for OA. Firstly, there are analgesics which are pain relievers that include acetaminophen and opioids. Secondly, there are nonsteroidal anti-inflammatory drugs (NSAIDs). These are used to ease inflammation and they include aspirin, ibuprofen, naproxen and celecoxib. Counterirritants are used to irritate

nerve endings causing the painful area to feel cold, warm or itchy which takes focus away from the actual pain. Corticosteroids are anti-inflammatory medicines that work similarly to the cortisol hormone. Hyaluronic acid injections are also used in OA treatment. This helps to reduce or relieve inflammation as the gel aids in restoring lubrication and inducing the growth of new cartilage and bone tissue. Also, platelet-rich plasma (PRP) is used in treatment as it contains proteins that help to ease pain and inflammation. Lastly, other drugs such as the anti-depressant duloxetine and the anti-seizure drug pregabalin are used to treat OA pain too (Arthritis Foundation, n.d.).

Non-drug therapies include exercise, weight loss and surgery. Exercise is important as movement is essential in OA treatment. Strengthening exercises help to build muscle around the painful joints and ease the stress. Range-of-motion exercise or stretching help to reduce stiffness. Aerobic and cardio help improve stamina and energy levels and reduce stress. Lastly, balance exercises help strengthen small muscles around knees and ankles and help prevent falling which would only exacerbate the injury. Losing weight helps reduce pain as well as stop or slow down joint damage. For every pound lost, four pounds of pressure is removed on lower-body joints. Finally, as mentioned before, surgery is a form of treatment, but it is a last resort in late-stage OA when no other treatment options will aid in OA management (Arthritis Foundation, n.d.).

2. RESEARCH OVERVIEW

2.1. Aims of the study

The general aim of this study was to study to the transcriptomic profile of EVs in SF and blood of OA patients in order to define potential biomarkers for early OA diagnosis. The specific objectives of my research were:

- To give an overview of the current knowledge about OA transcriptomic profile.
- To give an overview of the OA studies regarding EVs derived from tissues, more specifically SF and blood derived EVs of OA patients.
- To further analyse the role of miR-146a and miR-26b in OA.
- To evaluate the potential of using EVs as biomarkers for OA diagnosis.

2.2. Materials and methods

PubMed (*PubMed)* was the main database used for research on this topic. All used articles were written in English. Key words used to find articles were "extracellular vesicles" and "osteoarthritis". These key words were used both separately or combined with "blood", "serum", "plasma", "synovial fluid", "joint fluid" "transcriptome", "exosome", "microvesicle", "apoptotic bodies", "extracellular RNA", "RNA", "miRNA", "miR-146a", "miR-26b".

Some miRNA target genes were found in articles while other target genes and their respective target scores were found through the use of MicroRNA Target Prediction Database (*MiRDB - MicroRNA Target Prediction Database*). According to MiRDB, a predicted target with a prediction score of 80 or higher is most likely to be real. If the score is below 60, then there is a need for caution, and it is recommended to have supported evidence. As well as using MiRDB to find target genes, TargetScan Database (*TargetScanHuman 7.2*) was used to confirm the miRNA targets.

2.3. Results and discussion

2.3.1. General OA transcriptome profile

At least one publication was found for each tissue listed in Table 2 (p.31) that carried out an in-depth analysis of the OA transcriptomic profile. However, if more than one publication was found regarding the same tissue, the most recent article was used.

Dysregulation in the transcriptome leads to the differential expression of genes which contributes to the pathogenesis of OA. These differentially expressed genes (DEGs) have been found in various OA tissues such as the cartilage, chondrocytes, subchondral bone, meniscus and synovial tissue which is shown in Table 2 (p.31). The most notable dysregulation of RNAs occurs in chondrocytes and this dysregulation can lead to chondrocyte apoptosis. Chondrocyte apoptosis is one of the key features of cartilage degradation, which in turn is a key characteristic of OA. There has been shown to be a significant positive correlation between the severity of OA and the number of apoptotic chondrocytes in OA cartilage ($p = 0.016$) (Zhong *et al.*, 2016). Therefore, it is possible to conclude that the tissues with higher amounts of DEGs are more affected by the disease.

Table 2. The dysregulation of RNA species in various OA tissues. up – up-regulated RNAs; down – down-regulated RNAs; PBMC – peripheral blood mononuclear cells; dmg – damaged regions; undmg – undamaged regions.

Tissue	Method of Analysis	Sample size	RNA species	Dysregulation	Ref
Cartilage	RNA-seq, qPCR	9 (all OA; dmg vs. undmg)	mRNA	401 up 338 down	(H. Li *et al.*, 2019)
Meniscus	Microarray, qPCR	24 (12 OA, 12 non-OA)	mRNA	75 up 93 down	(Brophy *et al.*, 2018)
Synovial tissue	qPCR	50 (22 OA, 28 non-OA)	mRNA	145 up 84 down	(Z. Li *et al.*, 2019)
Subchondral bone	Microarray, qPCR	25 (20 OA, 5 non-OA)	Total RNA	420 up 552 down	(Chou *et al.*, 2013)
Articular chondrocytes	Microarray	20 (12 OA, 8 non-OA)	Total RNA	888 up 732 down	(Aki *et al.*, 2018)
PBMC	Microarray	41 (19 OA, 22 non-OA)	mRNA	791 up 440 down	(Shi *et al.*, 2019)
Serum	qPCR	24 (12 OA, 12 non-OA)	miRNA	205 up 74 down	(Ntoumou *et al.*, 2017)

To further understand the effect that the dysregulation of RNA molecules has on OA pathogenesis, DEGs with the highest significance according to the publications listed in Table 2 were studied.

Table 3. The top five DEGs found in OA tissues and the affected pathways, which were chosen based on either their p-value or what the authors of the article emphasised. The number of DEGs can be seen in Table 2. GO – gene ontology. RA – rheumatoid arthritis. PPAR – peroxisome proliferator-activated receptors. AMPK – AMP-activated protein kinase. MAPK – mitogen-activated protein kinase.

Tissue	Top DEGs	Pathways	Ref
Cartilage	*WISP2, ATF3, CHI3L1, ADAM12, COL3A1*	ECM-receptor interaction, TGF-β signalling, osteoclast differentiation, insulin signalling	(H. Li *et al.*, 2019)
Meniscus	*CSN1S1, COL10A1, WIF1, SPARCL1, VEGFA*	Response to external stimuli, regulation of inflammatory response, immune system, response to wounding	(Brophy *et al.*, 2018)
Synovial tissue	*ADIPOQ, IL6, CXCR1, IL8, CXCL1*	Cytokine-cytokine receptor interaction, chemokine signalling pathway, RA, TNF signalling pathway, PPAR signalling pathway, AMPK signalling pathway	(Z. Li *et al.*, 2019)
Subchondral bone	*COL3A1, TUBB3, PPEF1, DIO2, TNSFS11*	Lipid and mineral metabolism, connective tissue disorders, cellular growth and proliferation	(Chou *et al.*, 2013)
Articular chondrocytes	*COL2A1, ASPN, IGFBP7, FOXO1, COL3A1*	ECM-receptor interaction, focal adhesion, protein digestion and absorption pathways	(Aki *et al.*, 2018)
PBMC	*RPL38, P1K3CD, MAPK14, IL1A, JUND*	Osteoclast differentiation pathway, MAPK signalling pathway	(Shi *et al.*, 2019)
Serum	*INSR, IGF1R, CREB5, NFκB, TNFα*	ECM-receptor interaction, TGF-β signalling pathways, Wnt, FoxO, mTOR, pI1K/akt, MAPK	(Ntoumou *et al.*, 2017)

It has been demonstrated that in OA pathogenesis the expression of genes that participate in certain pathways has been altered and this affects the functionality of the pathway. One of the affected pathways is the ECM-receptor interaction pathway and this has been demonstrated in the cartilage, articular chondrocytes and serum (Table 3). Structural matrix macromolecules in

the ECM and growth factors regulate chondrocyte functions through specific membrane receptors. DEGs such as collagen type II alpha 1 chain (COL2A1) (chondrocytes, Table 3) and COL3A1 (chondrocytes and cartilage, Table 3) are involved in the ECM-receptor interaction pathway. COL2A1 takes part in the metabolic activation of OA (J. Sun *et al.*, 2015) while COL3A1 is important in cartilage function (He *et al.*, 2016) and has been shown to correlate with radiographic severity of canine elbow OA (Clements *et al.*, 2009). Therefore, based on these findings it is clear to see that that the ECM-receptor interaction is important in chondrocyte functionality and any alterations in this pathway can contribute to the pathogenesis of OA.

Another pathway affected by the dysregulation is TGF-β signalling as well as the osteoclast differentiation pathway. TGF-β signalling has been shown to occur in the serum as well as the chondrocytes in cartilage (Table 3, p.32). *ASPN* in chondrocytes codes for a protein named asporin which is a component in the ECM. Asporin binds to collagen fibres to induce mineralization and it also inhibits TGF-β induced cell type differentiation (Aki *et al.*, 2018). TGF-β plays a role in the development and homeostasis of tissues by regulating proliferation, differentiation, apoptosis and migration, as well as controlling ECM synthesis and degradation (Blaney Davidson *et al.*, 2007). Furthermore, osteoclast differentiation was observed in the cartilage and PBMCs (monocytes differentiate into osteoclasts). Osteoclasts are cells that degrade bone during bone remodelling in both normal and pathologic states (Boyce *et al.*, 2009). The differentiation, activity and survival of osteoclasts is highly regulated in non-OA individuals but in OA patients, osteoclastogenesis is increased, resorption is elevated, and apoptosis of osteoclasts is reduced (Löfvall *et al.*, 2018). Therefore, it is evident that osteoclast differentiation and TGF-β signalling also play an important role in OA pathogenesis. From this it is possible to conclude that the altered expression of certain genes affects the functionality of pathways which, in turn, contributes to OA progression.

It is evident that these previously discussed changes in gene expression have contributed to the pathogenesis of OA. Despite this, there is no correlation between the SF and plasma miRNA profile (Murata *et al.*, 2010). Therefore, it is not possible to compare the transcriptomic profile of the two for OA diagnostic purposes. However, since EVs are capable of transporting and protecting their RNA cargo, the transcriptomic profile of EVs from OA tissue could possibly be used in OA diagnostics. We hypothesized, that the EVs could be migrating from the SF into the peripheral blood and through comparing miRNAs in both the blood and SF, it could be possible to determine a set of miRNAs that could act as a specific

OA biomarkers. This would prove to be a much more effective way of diagnosing OA taking a blood sample is much easier and less invasive compared to collecting SF.

2.3.2. EVs derived from OA tissues

The amount of publications to be found on the OA-EV transcriptomic profile is very small and only one article was found that provided an in-depth analysis of the SF-EV profile. Furthermore, two other articles were found, one regarding the exRNA profile in SF and another regarding EVs in plasma. The EV transcriptomic profile is important as we think it might be possible to compare SF and blood EV-miRNA profile for OA diagnostics. It has already been shown that the expression patterns of exosomal miRNA can differ from intracellular miRNA. These miRNAs were isolated from MC/9 cells, human mast cells and bone marrow mast cells. They found that some of the miRNAs were expressed at higher levels in exosomes than in cells (Valadi *et al.*, 2007). This combined with the fact that EV-miRNA content varies between OA and non-OA patients (Esa *et al.*, 2019; Kolhe *et al.*, 2017), there is a possibility that EV-miRNAs can be used as OA diagnostic tools.

Table 4 (p.35) shows the dysregulation of different exosome derived or extracellular RNAs (exRNA) in SF and plasma. When comparing Table 4 (p.35) and Table 2 (p.31), it is clear to see that in the case of OA the number of RNAs dysregulated in solid tissues is much higher than the RNAs dysregulated in EVs from SF and plasma. If a miRNA subtype can be found to be similarly regulated in both solid tissues and SF and plasma, it could prove to be a possible biomarker for OA. For this reason, it is important to find specific RNA molecules that are similarly regulated in both OA tissue and in EVs derived from OA tissue. However, if the content of EVs derived from OA tissue and non-OA tissue varies, then it does not matter if the content matches the RNA profile inside the cells. The difference in the EV-RNA profile can be used as biomarkers. Therefore, first the content of EVs dervied from OA and non-OA tissue should be analysed first and then decided if a comparison to the RNA profile of cells is needed.

It is also worth noting that the study done by Aae *et al.* (Table 4, p.35) evaluated plasma EV-miRNAs as possible biomarkers for OA but the levels of plasma EV-miRNAs were similar for all participants, OA and non-OA alike. However, this study focused on the general EV-miRNA profile rather than on specific miRNA subtypes that could be potential biomarkers for the disease. As well as this, they focused on EVs in general which means that this study evaluated the exosome, MV and AB miRNA content rather than just focusing on the exosomal miRNA content as most studies do. Also, the miRNA cargo within exosomes seems

to be gender specific (Kolhe *et al.*, 2017) and this was not a factor considered in the study done by Aae *et al.* Therefore, it could still be possible to find biomarkers for OA in plasma.

Table 4. The dysregulation of RNA populations in EVs and their method of analysis. up – up-regulated; down – down-regulated; m – male; f – female; NGS – next generation sequencing.

Tissue	Method of Analysis	Sample size	Origin of RNA	RNA species	Dysregulation	Ref
SF	Microarray, qPCR	51 (33 OA, 18 non-OA)	Exosomes	miRNA	45 up, 68 down (m) 53 up, 91 down (f)	(Kolhe *et al.*, 2017)
SF	Total RNA-seq	19 (14 knee trauma, 5 healthy)	Extracellular	miRNA mRNA	3 up, 5 down 44 up, 25 down	(Griswold *et al.*, 2018)
Plasma	NGS	46 (23 OA, 23 non-OA)	EVs	miRNA	-	(Aae *et al.*, 2020)

Murata *et al.* have already discovered that the expression patterns of extracellular miRNAs in OA-SF were similar to miRNAs secreted by synovial tissues (Murata *et al.*, 2010). Based on Table 4 it is possible to see that the number of differently expressed miRNAs in exosomes is much higher than that of extracellular RNA (exRNA) in the SF. The lower number of exRNA populations could be explained by RNA degradation in the SF while exosomal miRNAs were protected from it. This shows that investigating exosomal miRNA is much better for the purpose of our study as the likelihood of obtaining intact RNAs is higher. Furthermore, it could be possible to compare the whole miRNA content with EV-miRNA content. Murata *et al.* believed that plasma miRNAs are generated by various tissues (Murata *et al.*, 2010) which could include synovial tissue. Therefore, based on previous research done on the topic, we can hypothesize that some of the SF-EVs derived from cartilage will potentially be transported into the peripheral blood. However, how slow or fast this migration could occur is unknown. As I mentioned before, one of the possible reasons why Aae *et al.* did not detect any differences in the plasma EV-miRNA content for early-stage OA and non-OA patients is

because not enough miRNAs had migrated yet. Nevertheless, this does not mean that it is not possible to find differences in expression at later stages of the disease.

Table 5. The presence of specific miRNAs in SF-EVs, and the possible origin of SF-EVs in OA patients. This table was composed by comparing a list of miRNAs believed to partake in OA pathogenesis (Panagopoulos and Lambrou, 2018) with a list of gender specific miRNAs found to be significantly dysregulated in SF-EVs (Kolhe *et al.*, 2017). Only miRNA molecules present in both studies were included. m – male; f – female; down – down-regulated; up – up-regulated.

miRNA	Tissue	Origin	EVs	Ref
miR-23a-3p	Cartilage SF	Chondrocytes Cells in SF	SF-EVs (f, down)	Kang *et al.*, 2016 Y.-H. Li *et al.*, 2016
miR-24	Cartilage SF	Chondrocytes Cells in SF	SF-EVs (f, down)	Philipot *et al.*, 2014 Y.-H. Li *et al.*, 2016
miR-26a	Cartilage	Chondrocytes	SF-EVs (f, down)	Rasheed *et al.*, 2016
miR-26b	Cartilage	Chondrocytes	SF-EVs (m, up)	Hu *et al.*, 2018; Xuefeng Yin *et al.*, 2017
miR-146a	Cartilage Cartilage	Chondrocytes Chondrocytes	SF-EVs (f, down)	Yamasaki *et al.*, 2009 J. Li *et al.*, 2012

However, the purpose of this study is to give an overview into the current knowledge on the transcriptomic profile of EVs found in SF and blood in the case of OA. As the blood profile of EV-miRNAs in OA is not yet well studied, more emphasis will be placed on SF EV-miRNAs and their potential in OA diagnosis. This will be done through determining if the chosen miRNA molecule has been analysed in the blood and if its expression can be related back to its expression in OA tissue and SF-EVs. If this comparison is successful, then it might also be possible that the expression of the blood EV-miRNAs will be similar due to migration from the SF into the peripheral blood. This migration is made possible by the phospholipid bilayer that surrounds EVs and protects their cargo. From Table 5, it is clear to see that miRNAs found in chondrocytes have also been found in SF-EVs and exRNAs have been found in the SF. Therefore, it is highly likely that some of the miRNAs found in SF-EVs could originate from chondrocytes.

Several miRNAs have been found to play a role in OA pathogenesis and Table 5 reflects a small portion of them. This table was narrowed down to the miRNAs that were also found to be expressed in SF-EVs. miR-23a-3p is thought to contribute to OA progression by targeting SMAD3 and thus, affecting the levels of type II collagen and aggrecan (Kang *et al.*, 2016). miR-24 has been shown to be a negative regulator of a senescence marker $p16^{INK4a}$. Overexpression of INK4a in chondrocytes induced the production of MMP1 and MMP13 which lead to senescence being linked with OA pathogenesis (Philipot *et al.*, 2014). Lastly, miR-26a has been shown to be a direct regulator of inducible nitric oxide synthase (iNOS) expression which has been associated with the pathogenesis of OA (Rasheed *et al.*, 2016). The rest of this study will focus on analysing the possibility of miR-146a and miR-26b being used as biomarkers for diagnosing OA.

2.3.3. miR-146a and miR-26b in OA

No miRNAs were found to be dysregulated in both male and female OA patients SF-EVs that have also been studied in OA pathogenesis so instead, focus will be turned to one miRNA found to be dysregulated in female OA patients SF-EVs and one miRNA dysregulated in male OA patients SF-EVs. For this reason, miR-146a and miR-26b were chosen. miR-146a was found to be significantly ($p = 0.00065$) dysregulated in only female patients' SF-EVs while miR-26b was found to be significantly ($p = 7.02E-05$) upregulated in male SF-EVs.

A table was composed to help evaluate the possibility of using miR-146a and miR-26b as biomarkers through showing their predicted targets that have a known role in OA pathogenesis (Table 6). Through this, the possible role of these two miRNAs in OA pathogenesis will be demonstrated. Furthermore, based on this information we can give a preliminary evaluation if miR-146a and miR-26b are viable biomarkers for early OA diagnostics.

Table 6. miR-146a and miR-26b and their predicated targets. Some target genes were identified through articles (which are referenced) while others were identified through the use of MiRDB (*MiRDB - MicroRNA Target Prediction Database*, n.d.) and TargetScan (*TargetScanHuman 7.2*, n.d.) databases. I chose these targets as I could find publications that linked these target genes to OA.

miRNA	Target genes	Target score	Ref
miR-146a	MMP13	-	Yamasaki *et al.*, 2009
	SMAD4	-	J. Li *et al.*, 2012; TargetScan
	MMP16	89	X. Li *et al.*, 2011; MiRDB
miR-26b	KPNA3	-	Xuefeng Yin *et al.*, 2017
	KPNA2	95	MiRDB
	COL10A1	97	MiRDB
	PTGS2	96	MiRDB

2.3.3.1. miR-146a

miR-146a expression can be induced by toll-like receptor (TLR) agonists, TNF-α or IL-1β (Song *et al.*, 2017) and it has been associated with numerous pathologies. It has been shown to affect cancer, inflammation and the innate immune response. Overexpression of miR-146a causes inhibition of NF-κB signalling, FOXP3-induced cellular apoptosis and PDGF-stimulated tumour growth while downregulation has been shown to contribute to 5q-syndrome (bone marrow disorder that results in too many immature blood cells and too few normal mature blood cells). It has also been implicated in cardiovascular diseases. Upregulation of miR-146a has been shown to contribute to coronary artery disease, atherosclerotic plaque formation, and attenuation of proinflammatory stress in hyperlipidaemia (Paterson and Kriegel, 2017). However, miR-146a has also been shown to have therapeutic potential as exosomal miR-146a has been shown to contribute to the increased therapeutic effects of IL-1β-pre-treated MSCs on cecal ligation and puncture-induced sepsis (Song *et al.*, 2017). miR-146a seems to be a multifaceted RNA molecule but most studies have focused on its inflammatory effect and not much attention has been paid to the possible therapeutic potential of exosomal miR-146a.

In OA, the dysregulation of miR-146a seems to depend on the stage of the disease. Most studies have focused on the upregulation of miR-146a which seems to occur in early OA

while down-regulation seems to occur in late-stage OA. In OA patients' cartilage, miR-146a was upregulated in grade I OA (grade I = mild OA scored 0-5 according to a modified Mankin score) (Yamasaki *et al.,* 2009) while it was seen to be downregulated in grades II and III (grade II = moderate OA scored 6-10, grade III = severe OA scored 11-14). While the expression of miR-146a decreased in grade II, the expression of matrix metalloproteinase 13 (MMP13) increased. Some believed miR-146a to be a negative feedback regulator of MMP13 and while miR-146a expression seems to decrease in accordance with the levels of MMP13 expression, this is speculation and has not been proven (Yamasaki *et al.*, 2009). However, MMP13 has been shown to be expressed in OA cartilage and it has the highest activity in the degradation of cartilage-specific type II collagen (N.-G. Li *et al.*, 2011). As well as MMP13, MMP16 is another predicted target of miR-146a. Upregulation of miR-146a suppresses MMP16 which is an important activator of MMP2 (X. Li *et al.*, 2011). As miR-146a is down-regulated in stages II and III which means MMP16 is not suppressed anymore and therefore, its target MMP2 is active which consequently also affects the pain experienced during OA. This demonstrates that miR-146a might play a role in OA cartilage pathogenesis and OA pain-related pathophysiology.

miR-146a is also predicted to target Smad4 in chondrocytes from tibial plateaus and femoral condyles. Smad4 plays an important role in chondrocyte differentiation by inhibiting hypertrophy and cell apoptosis. Smad4 is required for the normal organization of the cartilage growth plate (J. Zhang *et al.*, 2005) and it's absence is characterized by reduced chondrocyte proliferation, increased hypertrophic differentiation, and apoptosis (J. Li *et al.*, 2012). Overexpression of miR-146a inhibited Smad4 protein levels while downregulation of miR-146a results in the upregulation of Smad4. Evidently, upregulation of miR-146a plays a role in OA pathogenesis.

The presence of miR-146a has also been proven in both serum and plasma samples. A study compared cartilage and serum samples from OA patients to samples from patients with femoral neck fractures with no radiological symptoms or hip joint pain. It was found that miR-146a was significantly overexpressed in OA patients' cartilage and serum with a positive correlation between the levels in both (Skrzypa *et al.*, 2019). miR-146a was also found to be overexpressed in the plasma of early-stage OA patients (Cuadra *et al.*, 2014) while the concentration of miR-146a in SF was lower than in plasma. In another study where plasma and SF samples from late-stage OA patients were compared it was found that miR-146a levels were significantly higher in rheumatoid arthritis SF than in OA-SF (Murata *et al.*, 2010). However, Guan *et al.* demonstrated that miR-146a is downregulated in late-stage OA patients

cartilage (Guan *et al.*, 2018) which is supported by another study (Yamasaki *et al.*, 2009). This shows that the expression patterns of miR-146a depends on the stage of the disease and that it is highly likely that this expression pattern is mirrored in the blood.

As mentioned before, miR-146a was found to be significantly down-regulated in female OA patients SF-EVs (Kolhe *et al.*, 2017). In this study the OA-SF samples were received from knee joints of patients undergoing total knee arthroplasty procedures. A specific OA stage (early or late) was not given but total knee arthroplasty is a clinical end stage treatment of OA therefore it is possible to conclude that these samples came from patients with late-stage OA. As these donors potentially had late-stage OA, it is very likely that miR-146a in SF-EVs are similarly dysregulated as the miR-146a found in OA blood and tissue samples. Therefore, it is likely that miR-146a is up-regulated in early-stage OA SF-EVs and down-regulated in late-stage OA SF-EVs.

Overall, miR-146a seems to be upregulated in early stage OA but down regulated in late stage. The upregulation in the early stages might be a compensating mechanism to avoid the degradation of cartilage. Thus, it is inhibiting MMPs to avoid degradation, but on the other hand it affects Smad4, which causes a malfunction in cartilage formation. It is evident that miR-146a could play an important role in OA pathogenesis and could also prove to be a possible biomarker for the disease, especially early-stage OA as it is upregulated then. As well as this, it has been detected in synovial tissue, plasma, serum, and SF-EVs. Plasma miRNAs originate from various tissues and EVs can transport intact RNA molecules due to their phospholipid bilayer. Therefore, it is possible that some of the EVs from the joint tissue have reached the peripheral blood. However, I do not believe that miR-146a would make a good general biomarker for OA in female patients. While it is quite clearly dysregulated in the case of OA in both blood, tissues and EVs, it has also been found to be dysregulated in other diseases such as cancer (Adami *et al.*, 2019), rheumatoid arthritis (Bae and Lee, 2018), and Alzheimer's disease (Ansari *et al.*, 2019). In the case of cancer, it was found to be dramatically overexpressed in primary gastric tumours but it's expression levels decreased in progressed tumours (Adami *et al.*, 2019). While it seems to be a good biomarker to show that there are some pathological processes activated in the organism, I do not believe that miR-146a from SF-EVs could be accurately used as a specific biomarker for OA in female patients.

2.3.3.2. miR-26b

miR-26b has been shown to be a potential therapeutic candidate of osteoporosis as it has been shown to promote bone marrow stromal cell osteogenesis (H. Hu *et al.*, 2019) and could also possibly represent an effective therapeutic strategy for liver fibrosis (L. Li *et al.*, 2019). As well as having therapeutic potential, it has also been shown to play a role in the immune response as the overexpression of miR-26b inhibited the replication of vesicular stomatitis virus by upregulating interferons (IFNs) (C. Liu *et al.*, 2018) and it has also been implicated in cancer.

miR-26b has been found to be downregulated in patients with OA (Table 5, p. 36)(J. Hu *et al.*, 2018). It is an miRNA molecule that is capable of suppressing karyopherin subunit alpha 3 (KPNA3) by targeting its 3'-untranslated region which mediates NF-κB p65 translocation (Xuefeng Yin *et al.*, 2017). NF-κB itself is a transcription factor that serves as a mediator of inflammatory responses as it induces the expression of pro-inflammatory genes. Deregulation of NF-κB activation contributes to the pathogenic processes of inflammatory diseases such as OA (T. Liu *et al.*, 2017).

Similarly, another miR-26b target, KPNA2 (Table 6, p.37) is believed to promote NF-κB activation by facilitating P65 nuclear translocation and accelerating OA catabolic events. KPNA2 was shown to be upregulated in OA patient's cartilage and this up-regulation was accompanied by the elevated expression of catabolic protein levels, and increased NF-κB P65 translocation. As well as this, loss of KPNA2 resulted in a decreased translocation of the p50/p65 NF-κB complex (R. Tao *et al.*, 2015, p. 2). Therefore, as miR-26b seems to target both KPNA2 and KPNA3, it is evident that the miRNA plays a role in OA pathogenesis.

Furthermore, another predicted target of miR-26b is COL10A1 which is important in endochondral bone formation and it is specifically expressed when chondrocytes undergo hypertrophy. However, altered expression of COL10A1 is accompanied by abnormal chondrocyte hypertrophy which occurs during OA (Gu *et al.*, 2014). It has been suggested that during OA, articular chondrocytes undergo differentiation, hypertrophy, and apoptosis which mimic the endochondral pathway which is shown by the up-regulation of COL10A1 and enhanced chondrocyte hypertrophy has been observed in OA cartilage (Lu *et al.*, 2014, p. 1). Therefore, it is possible that the downregulation of miR-26b leads to the upregulation of COL10A1 which, in turn, contributes to OA pathogenesis.

Another predicted target of miR-26b is prostaglandin-endoperoxide synthase 2 (PTGS2, also known as COX-2) which helps with the production of prostaglandin (PG) E2. PGE2 is known

to often cause pain and swelling during inflammation and regulate bone formation. Increased expression of PTGS2 in the subchondral bone has been shown to be associated with spontaneous OA but not traumatic OA (Tu *et al.*, 2019). Therefore, it is possible that the upregulation of PTGS2 in the subchondral bone induces OA-associated joint cartilage degeneration.

miR-26b has also been found to be downregulated in male OA patient's SF-EVs and it is quite clear that it could play a role in OA pathogenesis as demonstrated by the genes it is predicted to target. However, despite extensive research I could not find any articles that linked the presence of miR-26b in blood to OA leading me to believe that such a study has not yet been carried out. miR-26b is a very attractive marker for OA but its expression profile should also be observed in the peripheral blood to determine if it is a viable biomarker for the disease in male OA patients.

2.3.4. Potential of EVs as biomarkers for OA diagnosis

There is potential in using EVs as biomarkers for OA diagnosis but before such a thing becomes reality, further studies need to be carried out. These possible future studies are discussed in the following Conclusions chapter.

miR-146a is not a good specific biomarker for OA diagnosis in female patients as it has been implicated in many diseases and conditions. However, it does have some potential as a biomarker to observe the progression of the disease once OA has been diagnosed. As mentioned before, miR-146a seems to be upregulated in early OA and downregulated in late OA. If the expression patterns of the miRNA molecule could be mapped in detail throughout the course of OA progression and it is reflected in EVs, it might be possible to determine the stage of the disease using a blood sample.

However, miR-26b seems to be a viable biomarker for OA in male patients as it is evident that this miRNA molecule could play an important role in OA pathogenesis, and it has not been implicated in as many diseases as miR-146a. However, the expression patterns of this miRNA molecule have not yet been investigated in the human blood or in blood EVs. As such, it is difficult to evaluate how good of an OA biomarker this molecule could be.

Another miRNA molecule that has potential as an OA diagnostic tool is miR-504. This miRNA is not mentioned above as there is a lack of information to be found in the context of OA. However, it is the only miRNA molecule found to be dysregulated in both male and

female OA patients SF-EVs (Kolhe *et al.,* 2017). As such I believe that this miRNA molecule does have the potential to be a good biomarker for OA diagnosis.

However, as it appears that a detailed study on the EV-miRNA profile of the serum, plasma and peripheral blood in OA patients has not yet been performed, it is not possible to estimate how good these EV-linked miRNAs could be as biomarkers for early-stage OA diagnosis.

2.4. Conclusion

OA is a progressive, musculoskeletal disease affecting millions of people worldwide with a considerable, long term effect on their quality of life. There is no known cure, but many strategies have been devised to help manage the symptoms. However, if a non-invasive early diagnostic method could be discovered, the quality of life for those suffering from OA could be greatly improved. A novel potential early diagnostic method is comparing the transcriptomic profile of OA patient's SF and blood. The OA transcriptomic profile would help to demonstrate the numerous changes that occur in OA tissue, but the SF and blood transcriptomic profile have shown no known correlation to that of OA and therefore is not usable as a predictive diagnostic tool for now. For this reason, focus has shifted to EVs and the cargo they contain, specifically miRNAs. It is possible to compare the general OA miRNA profile to SF EV-miRNAs, but it is yet to be determined if these EV-miRNAs get transported into the peripheral blood and could be compared to plasma EV-miRNAs. However, this is certainly a possibility as EVs are capable of transportation and protecting their RNA cargo. miR-146a and miR-26b seem to undoubtedly play a role in the pathogenesis of OA but their potential to be used as OA specific biomarkers remains to be determined with, seemingly, miR-26b having a higher potential.

However, publications addressing the transcriptomic profile of EVs in SF and blood from OA patients are virtually lacking. Nowadays we have access to high throughput technology through which it is possible to carry out more detailed studies. With this kind of technology in mind, a potential study that could be done is to take samples from OA (with different stages of OA) and non-OA patients and study the transcriptomic profile of their blood, SF and solid tissues (cartilage, bone, etc). The focus should be on the general transcriptomic profile as well as the EVs and the EV-derived miRNA profile. Furthermore, focus should also be turned to the expression patterns of miR-504 as it has already been shown to be dysregulated in male and female OA patients. However, during these studies the samples should be divided into separate gender groups and analysed separately. Moreover, I feel that studies should be

focusing on one EV subtype (exosome, MV or AB) rather than EVs in general for better consistency and to prevent the possibility of false conclusions.

Ekstratsellulaarsete vesiikulite transkriptoomiline profiil osteoartriiti põdevate patsientide sünoviaalvedelikus ja veres

Kristen Orumaa

RESÜMEE

Osteoartriit (OA) on krooniline luu- ja lihaskonna haigus, mis mõjutab suurt osa vanemaealisest elanikkonnast. OA ei ole ravitav, küll aga on võimalik kontrollida selle haiguse kulgu. Kui oleks võimalik haigust diagnoosida varjases staadiumis, siis oleks võimalik tunduvalt rohkem parandada OA põdevate patsientide elukvaliteeti. Praegu diagnoositakse OA-d kliiniliselt (sümptomite põhjal), radioloogiliselt (näiteks osteofüütide olemasolu põhjal) või sümptomaatiliselt (valu sageduse ja radiograafilise tõendid põhjal). Varajast diagnoosi on raske panna, kuna OA algusstaadiumis radioloogilisi sümptomeid veel pole, seega võiks varajane diagnoos põhineda näiteks molekulaarsetel markeritel. Üheks potentsiaalseks varajase diagnoosi molekulaarseks markeriks võiks olla verest eraldatud ekstratsellulaarsetes vesiikulites (EV) leiduvate RNA-de muster. Sellest viidi antud töö raames läbi teaduskirjandusel põhinev uuring, mis annaks ülevaate teadmistest EV transkriptoomi profiilist OA põdevate patsientide sünoviaalvedelikus (SF) ja veres võrreldes OA-d mitte põdevate inimestega. Lisaks püüti leida tõendeid, kas SF EV-de transkriptoomi profiil peegeldub veres, mis võimaldaks seda varajase diagnostika tööriistana kasutada.

Uurimistöö käigus leitud kirjanduse põhjal pole SF ja plasma üldise transkriptoomi profiili vahel korrelatsioone leitud, seega ei ole hetkel võimalik plasmat analüüsides teha järeldusi SF kohta. Siiski enamik, kui mitte kõik bioloogilised vedelikud sisaldavad EV-sid ja EV-d on võimelised nii transportima kui ka kaitsma neis olevaid RNA molekule lagunemise eest. Uuringute käigus selgus, et mõned SF-is pärinevate EV-de miRNA-d, mis on seotud OA patogeneesiga, on tuvastatud ka üldises SF transkriptoomilises profiilis. Samuti leiti, et vere EV-de profiili kohta on tehtud väga vähe uuringuid, mistõttu ei ole võimalik lõplikult öelda, kas teatud miRNA-sid saaks kasutada biomarkeritena. EV-de profiili analüüsimine diagnostilisel eesmärgil omab suurt potentisaali, kuid et seda rakendada, tuleks antud teemal teha hulgaliselt lisauuringuid. OA kudedest tuvastatud miRNA-de hulgast keskenduti töös miR-146a-le ja miR-26b-le. Mõlemaid on seostatud erinevate haigustega ning on näidatud, et neil on ka teatav terapeutiline potentsiaal. Uurimustöö tulemuste põhjal võib järeldada, et kuigi miR-146a ei pruugi olla otstarbeks varajase OA diagnoosimisel, võib see olla kasulik OA progresiooni määramisel pärast haiguse diagnoosimist. miR-26b võib siiski osutuda

meespatsientide OA potentsiaalseks biomarkeriks, kuid see vajab veel põhjalikumaid uuringuid.

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