Surgical aspects and microRNA in knee cartilage pathology

PhD Thesis

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Populærvitenskapelig norsk sammendrag

Kirurgiske aspekter og mikroRNA ved knebrusk patologi

Bruskpatologi i kneleddet er forbundet med store helsekostnader, pasienter kan bli utsatt for pasientskader og man mangler en blodmarkør som kan påvise tidlig artrose.

I sin avhandling "Surgical aspects and microRNA in knee cartilage pathology" har Tommy Frøseth Aae og medarbeidere undersøkt kostnadene knyttet til 2 kirurgiske behandlingsalternativer for bruskskader i kneleddet, kartlagt klagesaker i Skandinavia etter slik kirurgi og undersøkt om sirkulerende mikroRNA kan brukes som en blodmarkør for å oppdage tidlig artrose.

De fant at begge kirurgiske behandlingsalternativer for bruskskader i kneleddet gav bedring i symptomer hos pasientene, men at den ene kirurgiske behandlingsmetoden (mikrofraktur) hadde betydelig lavere kostnader. Denne kunnskapen kan supplere den kliniske beslutningsprosessen.

Videre fant de få pasientklager etter kirurgisk behandling av bruskskader i kneleddet. Til tross for at mange blir operert for disse skadene, er det kun et fåtall som innsender klage på grunn av behandlingsfeil. Årsakene til dette er ikke kartlagt og bør undersøkes nærmere. Funnene belyser også nødvendigheten av å etablere et bruskkirurgiregister for å øke kunnskapen om kirurgisk behandling av bruskskader i kneleddet og forbedre pasientsikkerheten.

Til slutt fant de at sirkulerende mikroRNA ikke kan brukes som en blodmarkør for artrose, men den høye forekomsten av mikroRNA varianter er påfallende og bør belyses i framtidige studier.

Contents

Acknowledgements

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Abbreviations

ACI	Autologous chondrocyte implantation
AD	Arthroscopic debridement
CEA	Cost-effectiveness analysis
DE	Differential expression
ECM	Extracellular matrix
EVs	Extracellular vesicles
FCD	Focal cartilage defect
ICD-10	International Statistical Classification of Diseases and Related Health Problems 10th Revision
ICRS	International Cartilage Regeneration & Joint Preservation Society
KL	Kellgren-Lawrence
MF	Microfracture
MP	Mosaicplasty
MIAME	Minimum Information about a Microarray Experiment
miRNAs	MicroRNAs
MP	Mosaicplasty
MRI	Magnetic resonance imaging
MUST	Musculoskeletal pain in Ullensaker Study
NCSP	NOMESCO Classification of Surgical Procedures
NGS	Next generation sequencing
nt	Nucleotide
OA	Osteoarthritis
PROMs	Patient reported outcome measures
QoL	Quality of Life
RCT	Randomized clinical trial
TMM	Trimmed mean of M-values

Papers in the thesis

Paper I

Aae TF, Randsborg PH, Lurås H, Årøen A, Lian ØB. Microfracture is more cost-effective than autologous chondrocyte implantation: a review of level 1 and level 2 studies with 5 year follow-up. Surg Sports Traumatol Arthrosc 2018 Apr;26(4):1044-1052.
doi: 10.1007/s00167-017-4802-5.

Paper II

Aae TF, Lian ØB, Årøen A, Engebretsen L, Randsborg PH.
Compensation claims after knee cartilage surgery is rare. A registry-based study from Scandinavia from 2010 to 2015. BMC Musculoskelet Disord 2020 May 8;21(1):287.
doi: 10.1186/s12891-020-03311-4.

Paper III

Aae TF, Karlsen TA, Haugen IK, Risberg MA, Lian ØB, Brinchmann JE. Evaluating plasma extracellular vesicle microRNAs as possible biomarkers for osteoarthritis. Osteoarthritis and Cartilage Open 1 (2020) 100018. doi: 10.1016/j.ocarto.2019.100018

Paper IV

Karlsen TA, Aae TF, Brinchmann JE. Robust profiling of microRNAs and isomiRs in human plasma exosomes across 46 individuals. Sci Rep 2019 Dec 27;9(1):19999.
doi: 10.1038/s41598-019-56593-7.

The thesis at a glance

Paper	Research question	Materials and Methods	Main findings
I	What are the costs of microfracture (MF) and autologous chondrocyte implantation (ACI) at 5 years following focal cartilage surgery in the knee?	Review of level I and level II studies with minimum 5-year follow-up. 4 articles with 170 MF patients and 149 ACI patients were included. Decision trees were designed, and costs were calculated.	MF had lower total costs (€5150) than ACI (€14,941) and lower costs per point increase in clinical outcome scores.
II	Are patient compensation claims after debridement and MF more frequent compared to mosaicplasty and ACI following focal cartilage surgery in the knee?	All complaints filed to the Scandinavian registries from 2010 to 2015 were collected and analyzed. 103 complaints were identified.	Almost all complaints were filed after debridement (43) and MF (54). Infection (22%) was the most common reason for granted claims.
III	Are plasma extracellular vesicles (EVs) microRNA (miRNA) potential biomarkers for osteoarthritis (OA)?	23 matched pairs of patients with and without OA. Plasma EVs miRNA were compared using next generation sequencing (NGS) technique.	177 plasma EVs miRNA were detected, although there was statistically no significant difference between the two groups.
IV	What is the content of miRNAs and isomiRs in plasma EVs, and are there difference in the distribution of 3p and 5p arms?	46 patients with and without OA. miRNAs and isomiRs were sequenced and characterized using next NGS technique.	177 miRNAs and 1716 isomiRs were detected. Minimal differences in concentration were found but were not statistically significant. 3p strand was as prevalent as 5p strand.

Preface

Cartilage and its diseases have troubled humans for millennia.
Aristotle (384 – 322 BC) stated, "Cartilage is found where it is an advantage that the solid framework should be pliable and glutinous for the benefit of the flesh that surrounds them."[1].

The first recorded pathological findings in cartilage were by Giovanni Battista Morgagni (1682 – 1771) when he assessed the hip joint during an autopsy: "The head of the right os femoris was not rounded into a globular form: and was depress'd, and not cover'd by a smooth and white cartilage, but by one of a pale ash-colour: and, indeed, this cartilage was totally deficient in the posterior part of the head; so that the bone appear'd naked in that part, and form'd into many roundish and protuberant particles.'"[2]. In hindsight, the findings by Morgagni are indeed osteoarthritis (OA) in its more severe form.

However, it was William Hunter (1718 – 1783) who launched one of the most frequently used quotes in cartilage research when he stated: "An ulcerated Cartilage is universally allowed to be a very troublesome Disease; … when destroyed, it is never recovered"[3].

Since the days of William Hunter, much progress has been made. Isolated cartilage defects are treated with a broad spectrum of surgical techniques with improvement of symptoms and function, whereas symptomatic end-state osteoarthritis is treated successfully with total joint arthroplasty. Still, cartilage pathologies are troublesome, and many issues remain to be answered. This PhD thesis aims to add knowledge to some of these topics.

Introduction

Focal cartilage defects

Background

There are three types of cartilage, namely fibrous cartilage, elastic cartilage and hyaline cartilage. Hyaline cartilage, also named articular cartilage, covers the bony ends of synovial joints with up to four-millimeter-thick articular cartilage (Figure 1). This highly specialized connective tissue allows for frictionless articulation and acts as a shock absorber and distributes load-bearing forces[4]. But these highly specialized functions come with a cost. Articular cartilage is avascular and aneural and devoid of lymphatic drainage and nerves with very limited capacity of healing[5]. An injury or disease to the articular cartilage often causes permanent damage. Focal cartilage defects (FCDs), may trigger pain, joint swelling, stiffness, catching, locking and reduced function and are assumed to predispose to OA[5]. Patients with FCDs in the knee joint are reported to have as impaired quality of life similar to patients with end-stage OA planned for total knee arthroplasty[6].

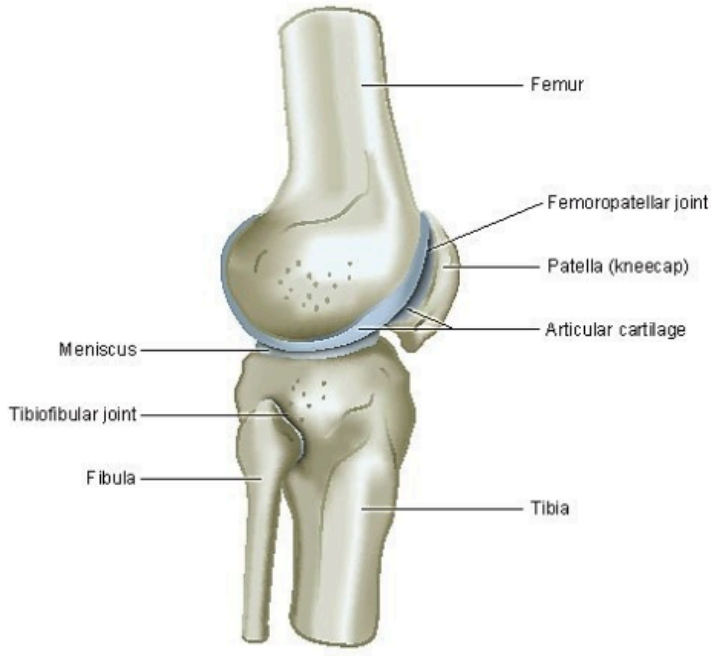

Figure 1. A schematic presentation of the knee joint, lateral view. The figure is reprinted with permission from International Cartilage Regeneration & Joint Preservation Society.

When FCDs are treated surgically, the aim is to restore cartilage, relieve symptoms and reduce the risk of OA[7,8]. The surgical treatment options span from simple debridement to advanced cell-based therapies and are performed in large numbers globally. In Norway, close to 2500 cartilage procedures are performed annually[9], and approximately 300 000 cartilage surgeries were performed in the United States in 2010[10]. Patients report less pain and better function following knee cartilage surgery, but normal knee function is generally not achieved[7,11,12]. Cartilage surgeries are associated with substantial costs both for the patient, the health care services and for the society at large connected to sick leave and maybe also disability. Furthermore, patients who undergo surgery can risk sustaining a treatment error which yields even higher costs related to these procedures. Patients who suffer a treatment error may file a compensation claims hereby further increasing costs following knee cartilage surgery.

The diagnosis of FCD is usually made based on a combination of the medical history, a thorough clinical examination combined with x-rays, magnetic resonance imaging (MRI) and/or arthroscopy[8]. When the diagnosis is made, the cartilage damage is already present, and very difficult if not impossible to reverse. Therefore, it is of interest to identify cartilage pathologies earlier, as this may reduce symptoms and prevent further cartilage destruction. Increased knowledge of cartilage pathologies may enable less invasive treatment options which most likely will reduce costs and treatment errors associated with cartilage surgery.

The present thesis aims to increase knowledge of cartilage pathologies, conducting studies focusing on costs and compensation claims following knee cartilage surgery and identifying a biochemical marker for early OA.

Articular cartilage morphology

The articular cartilage consists of cartilage cells (chondrocytes) and extracellular matrix (ECM). The chondrocytes develop, maintain and repair the main components of ECM except water. The chondrocytes are few; they constitute less than 5% of the volume of articular cartilage and scattered around in the ECM in lacunes with almost no potential of migration. The ECM is a highly organized network which provides the form and function of articular cartilage. The composition of ECM is mainly dependent on age. The main constituents of ECM are collagen, non-collagen proteins, proteoglycans and water. The former provides tensile strength and stretch resistance, whereas the non-collagen proteins provide resilience,

positioning within the ECM and adhesion. The proteoglycans add resistance to compression. These three components retain water in the ECM which constitutes approximately 80% of the weight of cartilage and is essential in maintaining the mechanical properties[5].

The articular cartilage is divided in four zones, all with unique composition and alignment providing different characteristics among the layers (Figure 2). The superficial zone has the highest concentration of collagen, the lowest concentration of proteoglycans and a rather high number of flattened chondrocytes. This zone is in direct contact with the synovial fluid and exhibits most of the tensile properties in cartilage. The middle zone contains a higher concentration of proteoglycans than in the superficial zone and the chondrocytes are few. This is the first zone involved in resistance to compressive forces.
The deep layer has the highest concentration of proteoglycans and water is depleted. This zone provides a resistance to compressive forces as collagen is orientated perpendicular to the joint surface. The fourth layer, the calcified zone, attaches the collagen to the subchondral bone.

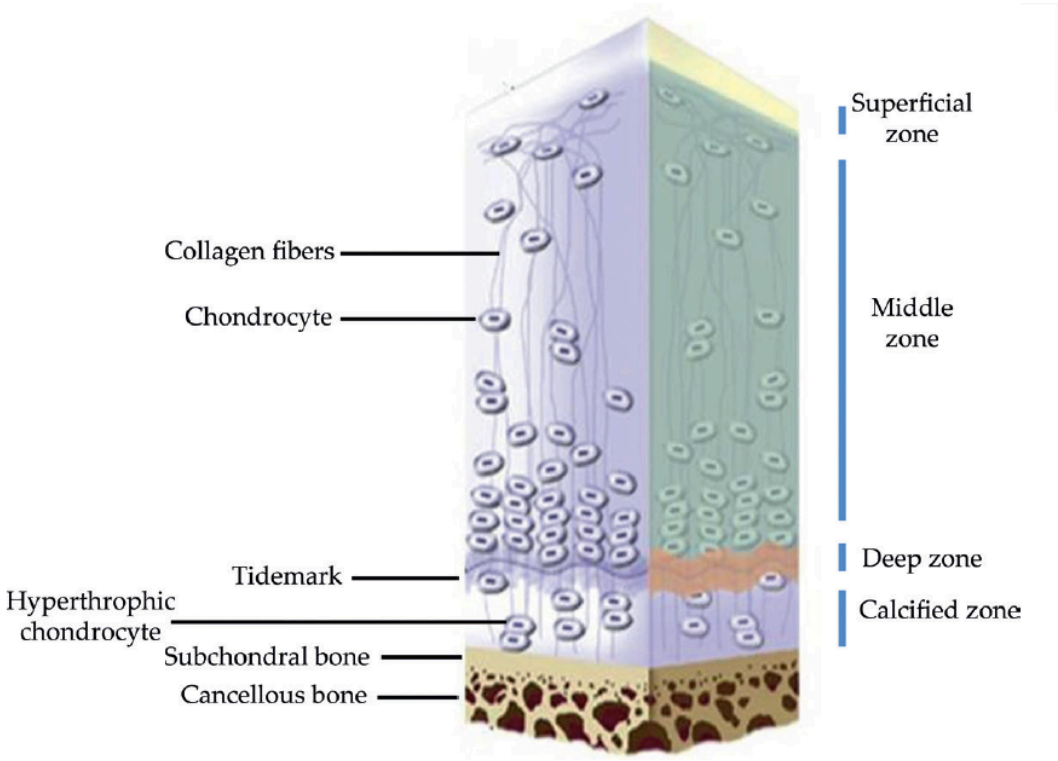

Figure 2. 3D structure of normal articular cartilage. The figure is explained in the text and is reprinted with permission from Moncada-Saucedo[13].

Cartilage pathologies are phenotypically either focal or degenerative[14]. Cartilage injury can occur after a single impact or after smaller, repetitive impacts, albeit the forces required to induce an injury to the cartilage are not fully understood[15]. If disruption of the superficial layer (and additional deeper layers) occurs, the homeostasis changes and the tightly controlled water distribution is impaired. This results in altered mechanical function of the cartilage. As the capacity for healing is low, even small superficial cartilage defects might not heal and may ultimately lead to degenerative changes of the cartilage[5].

Articular cartilage pathologies

Focal cartilage defects

A FCD is a clearly defined lesion/damage in articular cartilage of a given size which is surrounded by healthy cartilage (Figure 3). If the defect affects the underlying bone, the lesion is an osteochondral defect. Traditionally, FDCs are considered to be traumatic in origin, and can occur in all age groups. It is different from osteochondritis dissecans (OCD) which are lesions of unknown etiology that primarily affect children and adolescents[16]. OCD is a lesion of the articular surface that involves separation of a segment of cartilage from its underlying subchondral bone. This thesis will not cover OCDs in more detail.

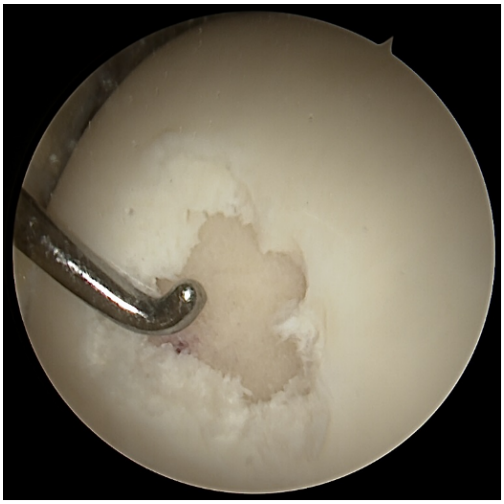

Figure 3. Arthroscopic visualization of a focal cartilage defect on the left.
Photograph by Per-Henrik Randsborg.

FCDs occur at all ages, but young adults and adolescent are most affected[17]. The true prevalence of FCDs is yet to be clarified, however, studies have estimated the prevalence to range from 12-66%[18,19]. Despite the high prevalence of FCDs, it is unclear if all are clinically

relevant or would require treatment[20]. Untreated FCDs are believed to eventually progress to OA, although this transformation is sparsely documented[20-22].

Osteoarthritis

Degenerative cartilage lesions are phenotypically different from FCDs and part of a continuum with early OA where a lesion also affects the surrounding and opposing cartilage. As the degeneration progresses to OA, the disease will gradually involve higher and higher proportions of the joint. End-stage OA not only affects the cartilage. Ligaments, tendons and muscles weakens, bony ends deform, synovium gets inflamed and the viscosity of synovial fluid is reduced[23]. OA is the most common adult joint disease and is now the leading cause of individual and societal health related costs[24-27].

OA can be divided into primary and secondary OA. In primary OA, also called idiopathic OA, there is no identifiable cause. Secondary OA is due to a known underlying condition. OA was initially considered a disease of age-related wear and tear on the cartilage, but later studies have revealed the etiology is much more complex[27]. The cartilage homeostasis is provided by a dynamic equilibrium between anabolic (such as insulin-like growth factors) and catabolic (interleukin-1, tumor necrosis factor and proteinases) influences[28]. A preponderance of catabolic factors in this equilibrium leads to ECM degradation. There is a broad range of risk factors for the development of OA[29]. Hereditary, gender and higher ages are known unchangeable risk factors of high importance for OA[27,30], whereas malalignment, cruciate ligament injury, meniscal tear and FCD all have been linked to secondary OA[29]. Obesity and repetitive activities in occupations are examples of modifiable risk factors associated with OA[31].

Symptoms

The clinical presentation of FCDs and early OA are similar. The dominant complaint is pain, but joint swelling, reduced mobility, stiffness, catching and locking may also be present[19]. However, cartilage pathology does not cause symptoms in all patients. Studies have reported asymptomatic cartilage injuries[19,20], and that only a minority of patients with radiologically verified knee OA complain of knee pain[32]. As cartilage is depleted of nerves, it is not the cartilage injury itself that elicit symptoms in FCDs and early OA. The reasons for this remain unanswered. Which conditions that needs to be present to cause symptoms is still unknown and beyond the scope of this thesis.

Diagnosis and classification

The diagnoses of FCDs and OA necessitates a thorough clinical examination combined with radiological assessment. It is not uncommon that FCDs are identified when searching for or being treated for another suspected knee injury. This is usually because initial symptoms are subtle and unspecific, and patients and physicians relate them to other diagnoses.

Radiography

Radiograph is the first examination to perform when searching for knee cartilage pathology. Radiograph is mandatory for the grading of OA and several classification systems exist. Weight-bearing radiographs are more sensitive than non-weight bearing x-rays[33]. The classification launched by Kellgren-Lawrence (KL) is the most used radiological classification system for knee OA[34]. The KL system is graded from 0 – none (absence of radiograph changes) to grade 4 – severe, with large osteophytes, marked joint space reduction, severe sclerosis and definite deformity of bony ends. OA is defined as present in KL grades 2 – 4. For all radiograph classification systems, FCDs and early OA are not easily detected in plain radiographs and supplementary radiological examinations with MRI are helpful.

MRI

MRI is a radiation free method that can offer additional information on other intraarticular injuries besides injury to the cartilage itself and has the potential to grade FCDs and OA according to localization, size and depth. MRI is considered objective and reproducible, yet the interpretation may be variable. In addition, sensibility and sensitivity vary among different MRI protocols[35,36]. This can lead to overlooked or misdiagnosis of FCD.

Arthroscopy

Arthroscopy offers a classification of both configuration and severity of FCDs. Although arthroscopy is an invasive procedure that potentially can lead to complications, it is considered the most accurate method to evaluate and grade an FCD.

There are three commonly used classification system for grading an FCD arthroscopically. The classification system designed by the International cartilage repair society (ICRS) is at presents the most widely used (Figure 4). This system grades a lesion's depth from grade 0 to grade 4. Grade 0 is normal cartilage, grade 1 is soft indentations, superficial lesions, fissures or small cracks, grade 2 is lesions extending down to less than 50% of the cartilage depth,

grade 3 involves more than 50% of the cartilage depth down to the subchondral bone plate. The most severe form, grade 4, involves lesion through the subchondral bone plate and down into the trabecular bone.

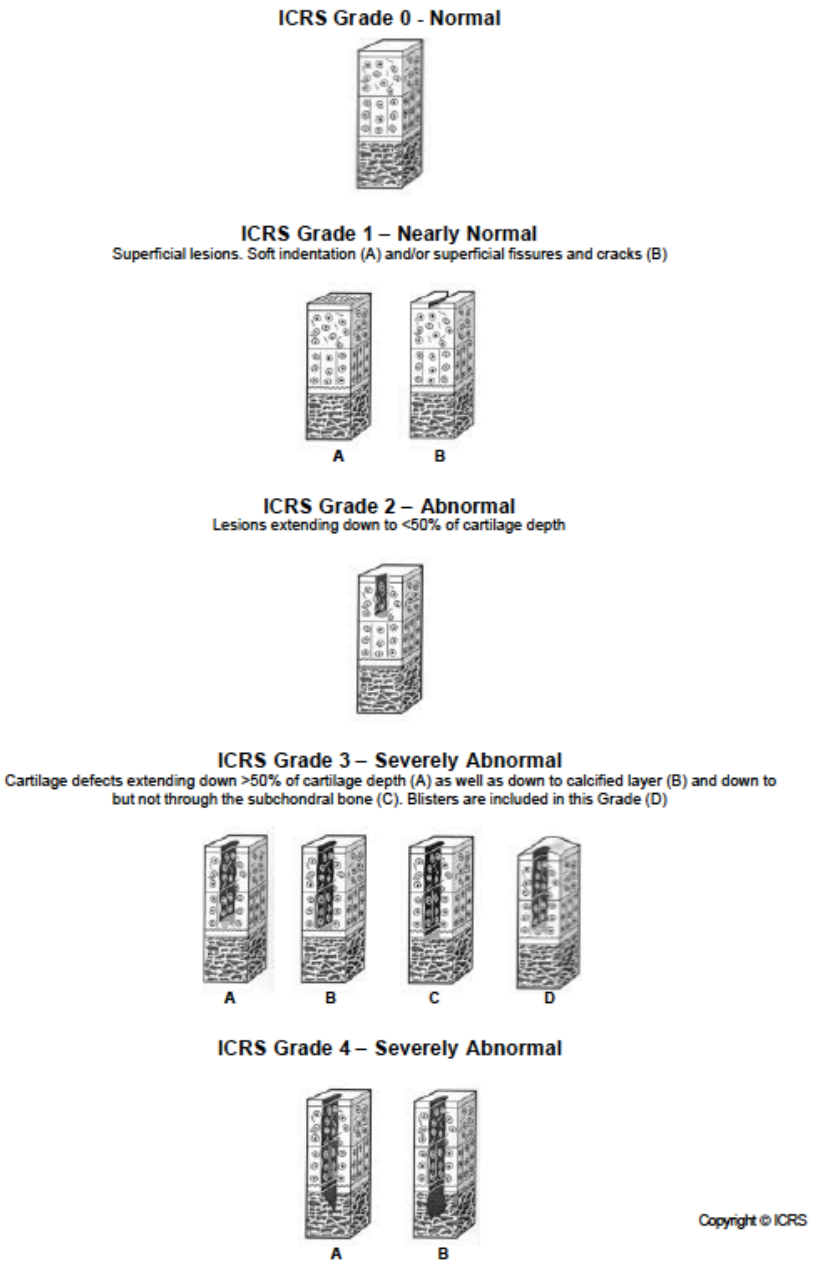

Figure 4. International Cartilage Regeneration & Joint Preservation Society (ICRS) classification system reprinted with permission from the ICRS.

Treatment options for FCDs

Non-surgical treatment options

Non-surgical treatment should be considered first choice in treating symptomatic cartilage lesions. The mainstay for non-surgical treatment is focused knee-strengthening exercises, preferably guided by a physiotherapist. Other non-surgical treatment options include lifestyle adjustments such as weight-loss and change of physical activity level, orthosis, pain killers, anti-inflammatory medication and intraarticular injections with hyaluronic acid, platelet-rich plasma or corticosteroids. For many of the surgical procedures available, both short- and long-term outcomes are well described in the literature[11,37-39], but for non-surgical treatment options, outcome and prognosis are rather sparsely addressed. However, the effect of physiotherapy on FCDs have gained more attention in recent years. One study found clinical improvement of full-thickness cartilage lesions following non-surgical and surgical treatment, albeit residual symptoms and development of secondary OA were registered for both treatment arms[40]. Dozin and colleagues reported that one out of three patients declined to participate in a clinical study comparing two surgical treatment options as symptoms were substantially reduced following 6 months of physiotherapy[41]. Another study reported that no surgical method is superior to rehabilitation alone[42]. Additionally, no report supports that physical activity worsens symptoms. On the contrary, physical activity have additive positive effects such as weight loss, beneficial gain in heart and lung diseases and prevents certain psychiatric diseases[43-45].

Surgical treatment options

The ideal treatment goal is to produce new hyaline cartilage with biomechanical properties resembling articular cartilage, to restore normal knee function and prevent development of early OA. However, such therapy is to date almost absent. Kjennvold et. al recently demonstrated that chondral fractures may heal if fixed acutely following injury and it is the only known way to save the hyaline cartilage after damage[46].

There is a broad range of surgical treatment alternatives for FCDs, from simple and low-cost procedures such as debridement and microfracture (MF), to advanced and expensive cell-based therapies including autologous chondrocyte implantation (ACI). Categorically, surgical treatment options can be divided in palliative techniques, repair techniques and restoration techniques, of which many can be combined with different additory such as scaffolds and matrixes (Figure 5). In the following, the four most used surgical techniques will be discussed in more detail.

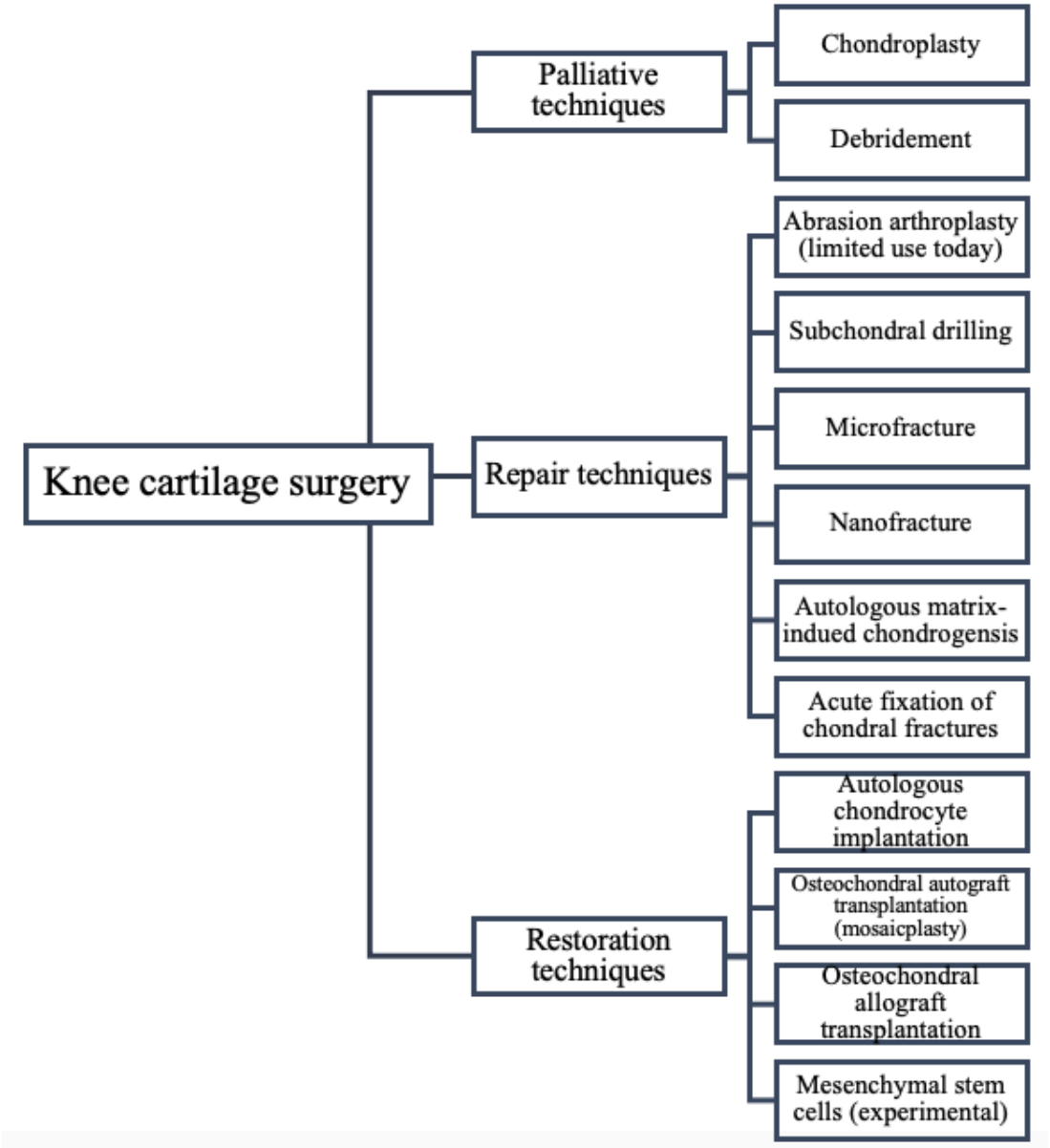

Figure 5. Overview of surgical treatment options for FCDs.

Arthroscopic debridement (AD)

AD is a simple one-stage palliative procedure aiming to reduce symptoms[47]. Loose cartilage flaps and fibrous tissue is removed down to the subchondral bone and the cartilage rim is stabilized to prevent further damage, but the cartilage defect itself it not addressed. AD is the most frequent procedure performed, mainly as a first-line treatment option, in patients with low-demand function as well in patients who suffer mechanical symptoms that inhibit rehabilitation[47,48].

Microfracture (MF)

MF is a one-stage repair technique and involves AD before the calcified layer is removed and the underlying subchondral bone is perforated (microfractured) with an awl[49]. By this method, multipotent cells are recruited from the bone marrow to produce a fibrocartilage filling of the defect (Figure 6). MF have been under thorough investigation in recent years. Good results compared to preoperatively have been demonstrated in patients younger than 40 years and for patients with a body mass index less than 30[50,51]. However, there is no randomized controlled trial comparing MF with AD or rehabilitation[52]. There is now an understanding that MF is not indicated for FCDs larger than 2 – 4 cm^2 in younger patients[53,54].

Figure 6. A schematic presentation of MF. The figure is reprinted with permission from International Cartilage Regeneration & Joint Preservation Society.

Osteochondral autograft transplantation (OAT)/Mosaicplasty (MP)

OAT is a one-stage procedure that can be performed arthroscopically or open. This technically demanding procedure involves transplantation of one or more osteochondral plugs into the defect harvested from a lower weight-bearing area in the knee following debridement (Figure 7). This is the only procedure which provides native hyalin cartilage to the defect

besides acute fixation of chondral fractures and OCDs[46]. OAT is termed mosaicplasty (MP) due to the mosaic appearance of the filled defect and in the reminder of this thesis, the term mosaicplasty will be used. MP is a treatment option for most FCDs and especially in defects involving the subchondral bone, although the technique is associated with donor-site morbidity[38,39,41].

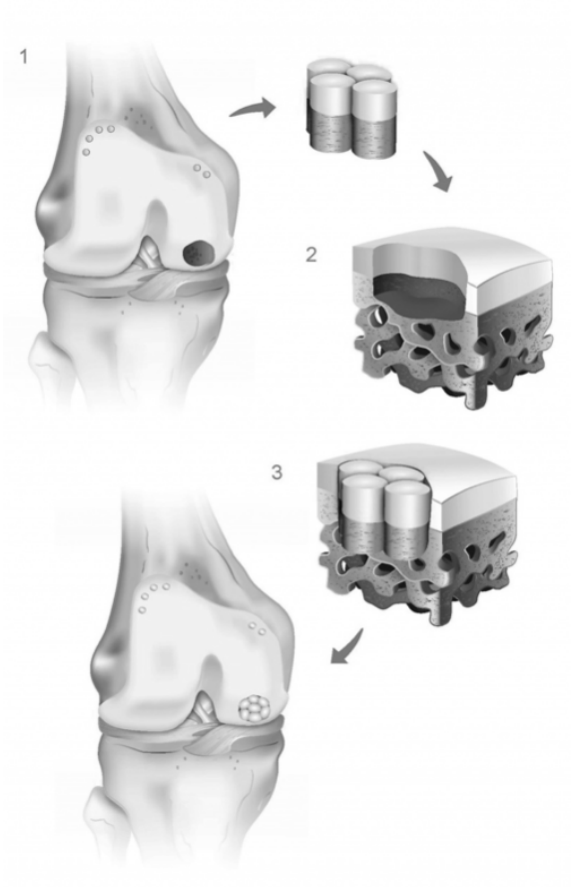

Figure 7. A schematic presentation of mosaicplasty. The figure is reprinted with permission from International Cartilage Regeneration & Joint Preservation Society.

Autologous chondrocyte implantation (ACI)

This mini-open two-step procedure first involves arthroscopic harvesting of small samples of normal cartilage, where extracted chondrocytes are cultured in the laboratory. The second part of the procedures involves debridement of the defects and reimplantation of the cultured cells into the defect maturing to hyalin-like cartilage (Figure 8). As for MP, ACI can be used to treat almost all defect sizes of FCDs but is dependent of a highly specialized laboratory for cell culturing[11,55].

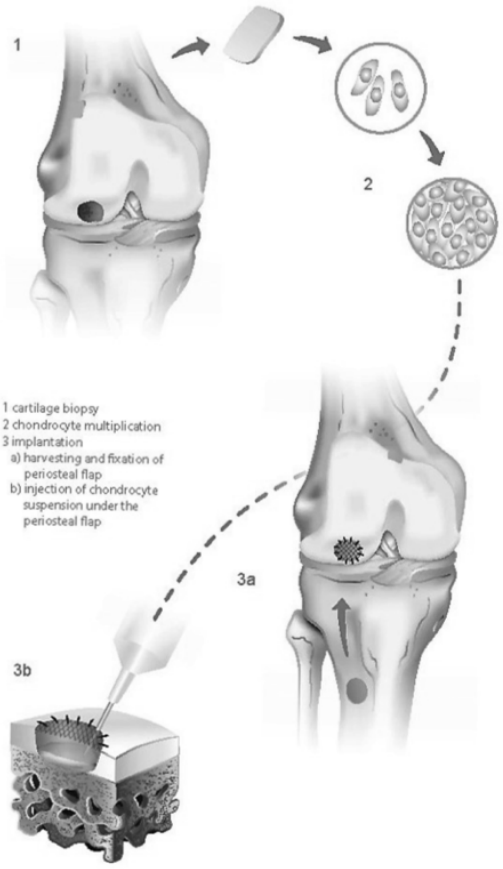

Figure 8. A schematic presentation of ACI. The figure is reprinted with permission from International Cartilage Regeneration & Joint Preservation Society.

Cartilage surgery is usually combined with postoperative rehabilitation. There is an extensive list of various rehabilitation protocols[56], and most authors agree that rehabilitation is an important factor for the clinical outcome following knee cartilage surgery.

Aspects of surgical treatment for FCDs

Clinical outcome

Cartilage surgery relieves symptoms regardless of procedure, but normal knee function is usually not restored[12]. Studies have revealed non-consistent clinical outcomes. AD has not been studied in controlled studies, but reports indicate that AD relieve symptoms especially in small FCDs[41,48].

Steadman et al. have published impressive results following MF for FCDs up to 10 cm^2. They reported that 83% had improved knee function at 7 years[37], but these findings are yet to be reproduced by others, and the study suffers from the lack of a control group[57]. Solheim et. al assessed 110 patients 10-14 years after MF and reported better knee function and less pain than preoperatively, but 39% were reoperated and 45% reported a poor outcome[12]. Knutsen et al. published long-term results comparing MF and ACI[11]. They found clinical improvement in both group at 14 – 15 years, but no difference in patient reported outcome measures (PROMs) or failures between the two groups. In fact, 32% in the MF group and 42% in the ACI group underwent reoperation and approximately half in each group developed radiologically OA[11]. Ulstein et al. have published a level II study with 5 – 11 years follow-up comparing MF with MP[38]. Their findings did not reveal differences in either PROMs, muscle strength or development of radiological OA. In a meta-analysis comparing MF and MP, Pareek reported favorable results following MP on activity level and lesion size > 3 cm^2 compared to MF[58]. Lastly, Horas et al. and Dozin et al. demonstrated better PROMs following MP compared to ACI[39,41], whereas Bentley et al. found the contrary and reported higher failure rates in the MP-group[55].

The various surgical treatment options have always been compared directly with each other in clinical studies. All cartilage procedures imply extensive postoperative rehabilitation and physiotherapy. Wondrasch et. al found that an active rehabilitation program for the treatment of FCDs improved symptoms and consequently rehabilitation and physiotherapy may partially explain the results demonstrated in clinical trials[42]. As far as we know, no randomized control trial has been conducted with a proper control group meaning that the true effect of surgery is in fact unknown.

To summarize, there is no consensus what constitutes the best surgical treatment option for FCDs. Given the high prevalence of FCDs, the lack of a superior surgical procedure and the increased focus on the continuously increasing health care costs, cost-effective analysis (CEA) can be useful as a contributor to the clinical decision process.

Cost-effectiveness

There are several methods to assess costs and effects related to health care interventions, all with pro and cons[59]. CEA are a commonly used method to evaluate costs and effects of health care interventions[60]. CEA establishes costs and impacts of health care interventions and aims

to improve efficiency, to gain more health for the resources available based on decision models[61]. Using a methodological approach, costs and effects are assessed and measured, thereby estimating the effectiveness of the intervention[60].

When costs are assessed, it is recommended that both costs related to the intervention and to the society are included, whereas effects are usually assessed to changes in PROMs. Rajan et. al have published a detailed checklist for reporting results of CEA in orthopedic surgery[62]. For FCDs, there is an increasing number of CEA evaluating the various surgical treatment options of which most are mainly based on short term follow-up[63,64].
Miller and colleagues compared the cost-effectiveness of MF and MP based on a literature search of level I and II studies[63]. They found that MF had lower initial costs, but the difference in costs lessened at 10 years. A cost-effectiveness review by Schrock et. al compared MF, MP and ACI[65]. They calculated a cost-per-point change in PROMs and reported that MF was the most cost-effective treatment options. Contrary to the findings by Schrock et. al, Misty et. al reported that ACI was more cost-effective than MF[66]. Samuelson et. al have examined the cost-effectiveness of ACI and reported that ACI were cost-effective and compared favorably with other surgical conditions[67]. Only one study has included the indirect costs to society related to sick leave[68].

In summary, studies reveal discrepancies in cost-effectiveness and demonstrate methodological variations in study design and follow-up period, and previous studies do not account for all costs related to the procedures such as preoperative imaging and postoperative rehabilitation. Hence, CEA for surgical treatment options for FCDs are inadequately described in the literature and further studies are needed to complement present knowledge.

Compensation claims

Despite every physician trying to follow the Hippocratic oath of "First, do no harm"[69], it is human to make mistakes. Patients who are treated may be exposed to treatment errors, which can lead to an injury that could have been avoided if the patient was treated according to current guidelines. If a patient suffers a complication due to a medical treatment error, the patient can file a compensation claim. There are two main compensation principles, a court-based fault system and a no-blame system[70,71]. In the former system, the liable is responsible for the costs of the claim based on a lawsuit and are handled by the juridical system[70]. This system is found in most jurisdictions in the United States and United Kingdom[72]. On the other

hand, a no-blame system eliminates the fault criterion as the insurance provider covers the costs of a claim[72,73]. These cases are usually handled outside the juridical system and are found in Scandinavia, France and New-Zealand[71,73]. An advantage of the latter system is that patient safety data are available for research and learning. Albeit both systems have pros and cons[71], the scope of this thesis is not to compare different compensation systems, and hence they will not be discussed in further detail.

Compensation claims in Scandinavia are handled by nationwide systems. In Norway, the complaints are handled by the Norwegian System of Patient Injury Compensation (NPE) and in Sweden, the National Swedish Patient Insurance Company (LöF) processes the compensation claims[74,75]. In Denmark, the Patient Insurance Association handles compensation claims regarding malpractice and injuries[76]. To be granted compensation in Scandinavia, three criteria must be met. Firstly, the treatment error must have occurred during examination, diagnosis, treatment (or lack of treatment) or during follow-up and must be deemed below standard or erroneous based on current treatment guidelines. Secondly, the injury must have resulted in a financial loss or to a permanent medical impairment of minimum 15%. Lastly, the claim must be filed within a reasonable time (currently 3 years in Norway and Denmark and 10 years in Sweden). There is one exception to these criteria, termed the exception clause. This clause opens for compensation to be given even if no treatment error has been identified if the injury is rare and severe. The compensation claims are reviewed on an individual basis and the amount of compensation is calculated to cover the loss of income and increased medical expenses due to the treatment error.

Orthopedic surgery is one of the leading medical specialties associated with compensation claims[72], accounting for 23% and 47% of all compensation claims filed to the Swedish and Norwegian registries respectively[77,78]. Most studies on compensation claims after orthopedic surgery to date have assessed compensation claims following arthroplasty surgery and spine disorders[79-81]. Only a few studies have published findings on malpractice litigation following arthroscopic surgery[82-84], but studies on compensation claims following surgical treatment of FCDs are to the best of our knowledge non-existent.

Treatment errors can have detrimental effect on outcome, patient safety and yield even higher health related costs. As knee cartilage surgery is performed at large numbers globally and with increasing incident (REF), an evaluation of compensation claims following knee

cartilage surgery may identify possible areas of improvement and hence, reduce treatment errors and costs related to these procedures.

A biomarker for early OA

Do we need a biomarker?

To identify cartilage pathology at an earlier stage could make it possible to take preventive measures to inhibit further cartilage damage and reduce symptoms, and hence, reducing costs and treatment errors. A potential biochemical marker for OA could fundamentally alter management, trials of therapies and the understanding of disease pathogenesis.
The definition of a biomarker is "a defined characteristic that is measured as an indicator of normal biological processes, pathogenic processes or responses to an exposure or intervention"[85]. This broad definition of a biomarker is distinct from clinical outcome assessment, as the former links a measurement to a prediction and the latter measure outcome that are important to the patient[86]. For the remainder of this thesis, the use of the term "biomarker" will refer to "a biochemical agent with diagnostic, monitoring and/or predictive characteristics".

An ideal biomarker should be a stable molecule little susceptible to modification, have disease specificity and sensitivity which can correlate with clinical evidence and should be measured by an easy and inexpensive method[87].

Molecular medicine has contributed significantly to the search of biomarkers for various diseases for the last few decades. We now have biomarkers for different types of cancer and heart disease[87]. The most suited biomarkers for early primary OA are believed to be molecules or fragments derived from collagen homeostasis or breakdown of bone and synovium found in either blood, urine or synovial fluid[88,89]. Aided by advances in imaging techniques and proteomics, several candidate biomarkers for OA have been launched. Studies so far have failed to identify a biomarker for OA, and the search for a biomarker is still ongoing.

MicroRNAs and their role in early OA

MicroRNAs are small double-stranded non-coding RNA molecules with length of 20 – 26 nucleotides (nt) and regulate gene expression. MiRNAs are found to be involved in numerous

biological processes and act as intercellular messengers[90]. MiRNAs have been associated with several human diseases such as various forms of cancer, neurological diseases and cardiovascular diseases[91,92].

The complex biogenesis of miRNAs involves multiple steps inside the cell nucleus and in the cell cytoplasm (Figure 9). In the cell nucleus, MiRNAs are first transcribed as primary transcripts to pri-miRNAs. Pri-miRNAs, usually > 100 nt long, are processed by the microprocessor complex consisting of the Drosha enzyme and two DGCR8 proteins into the 70 nt long pre-miRNAs.

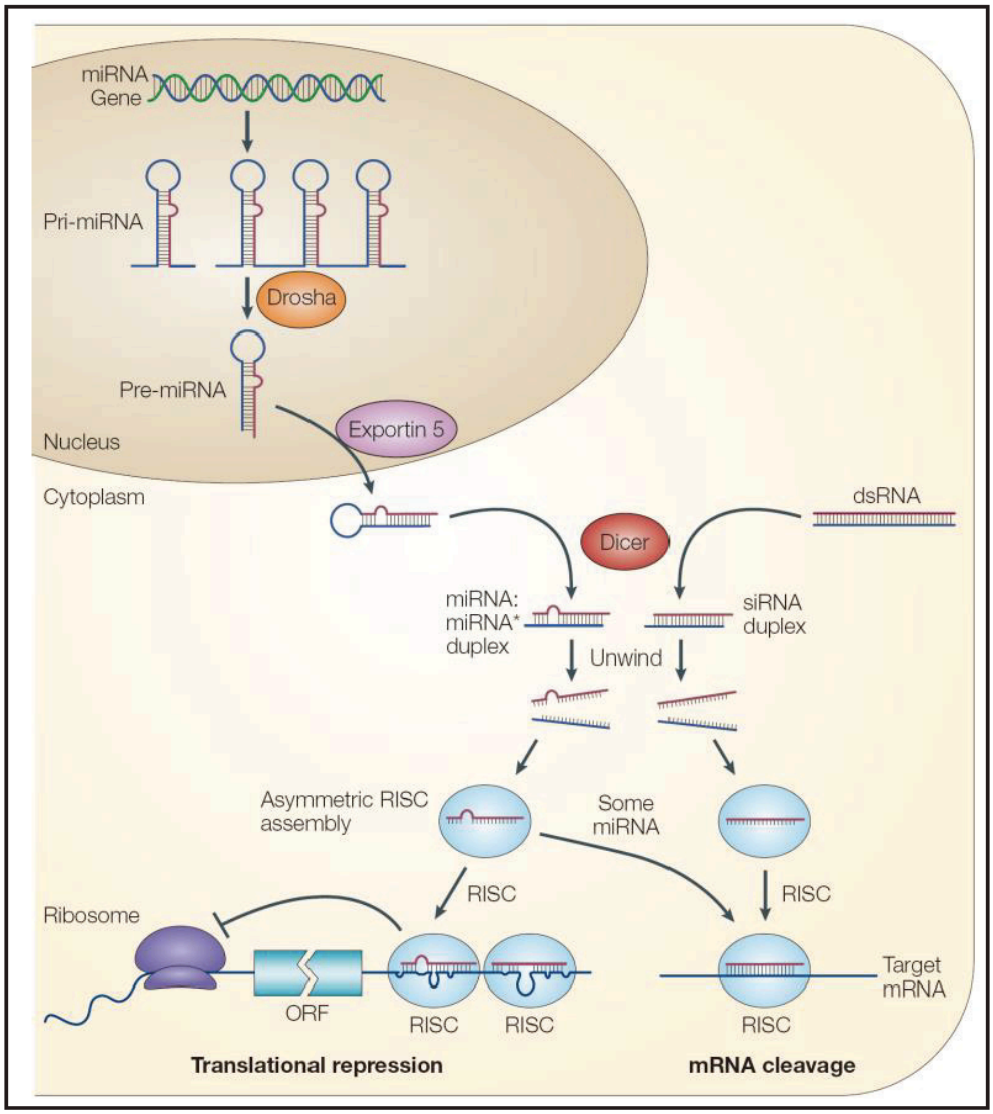

Figure 9. The biogenesis of miRNA. The figure is explained in the text and is reprinted with permission from Springer Nature[93], license number 4961241035270.

Following export to the cell cytosol, pre-miRNAs are cleaved by the Dicer enzyme into double-stranded miRNAs with a length of circa 20 – 22 nt. This miRNA duplex consists of two strands named the miRNA-5p (5p) and miRNA-3p (3p) and are held together with complementary base-pairing. Following Dicer processing, the two mature miRNA strands are loaded one at a time (termed the guide strand) into the Argonaute (AGO) protein.

Together with mRNA and several proteins involved in mRNA silencing or decay, the mature miRNA and AGO are referred to as the RNA-induced silencing complex (RISC). Inside RISC, miRNA will be processed and bind to complementary mRNA molecules and inhibit translation or degrade the mRNA (by the silencing effect)[94].

The classification of miRNAs is based on the unique sequence, and all miRNAs are listed in a global database (http://www.mirbase.org/). The most abundant read of a given miRNA in this database are labelled canonical miRNAs, whereas any variance in the unique sequence of canonical miRNAs are termed isomiRs. IsomiRs differs in sequence from the canonical miRNAs at the 5'end, 3'end or within the sequence and changes can either be addition or deletion of nt at the ends or substitutions within. The role of miRNAs have been under investigation for some time, although the distribution and role of isomiRs are still relatively unexplored[95].

To date, research on miRNAs as a potential biomarker for cartilage pathology are mostly studies of animals, synovial fluid and chondrocytes. Deletion of miR-140 in mice are found to predispose to OA-like changes[96], whereas miR-16-5p is reported to be expressed at higher levels in OA cartilage compared to healthy cartilage and assumed to contribute to the development of OA[97].

Extracellular vesicles

MiRNAs are largely found in the cell cytoplasm but are also found extracellularly where they are bound to proteins, lipoproteins or contained in vesicles[98,99]. Extracellular vesicles (EVs) are lipid bilayer particles that are released from the cell. The use of the term "extracellular vesicle" has been subject to discussion[100], as can be detected in papers III – IV. In paper III, the term EV was used, but in paper IV the term was exosome. For this thesis, EVs will be used. It is recognized that EVs are divided in at least 3 main classes; microvesicles, apoptotic bodies and exosomes[101] (Figure 10).

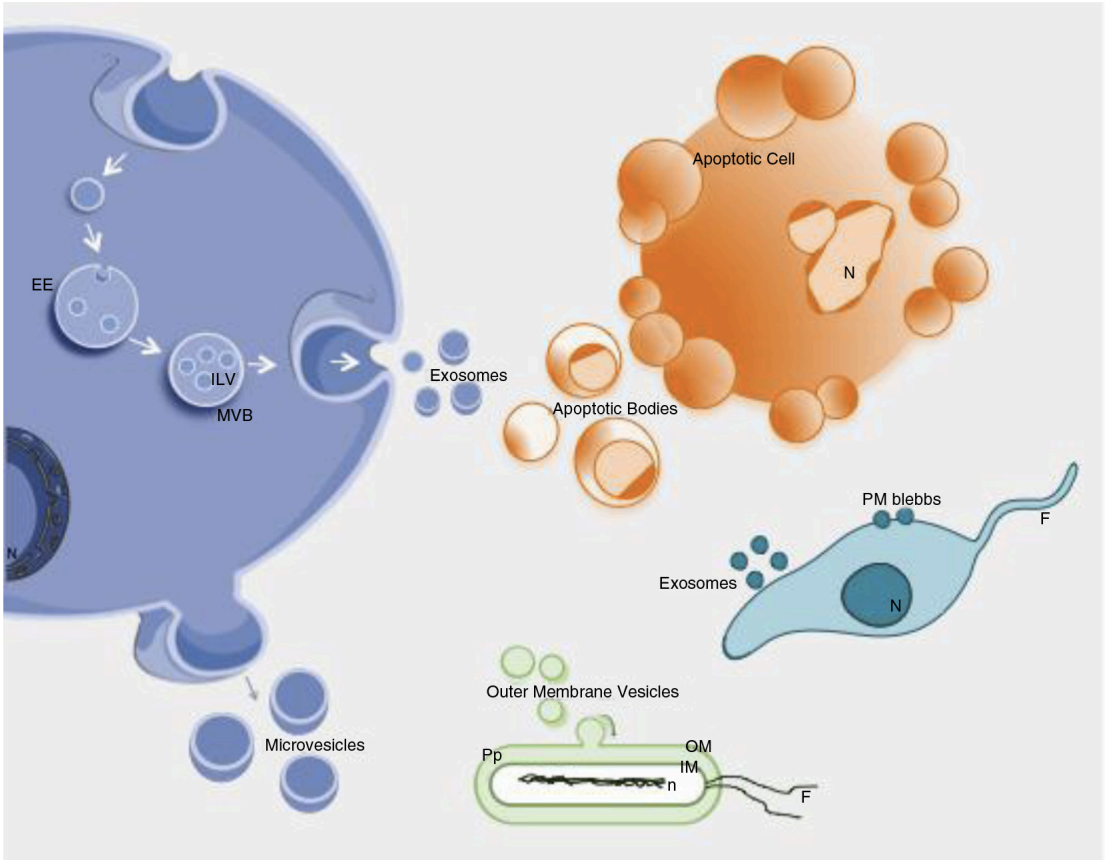

Figure 10. Schematic presentation of microvesicles, apoptotic bodies and exosomes. The figure is explained in the text and is reprinted with permission from Yáñez-Mó[101].

Microvesicles are heterogenous in size, ranging from 200 nanometer (nm) to greater than 1 µm in diameter and are released by outward budding of the plasma membrane, whereas the larger apoptotic bodies arise from cells undergoing apoptosis. Exosomes are small structures 40 – 100 nm and originate from intraluminal vesicles formed in clusters that fuses with the plasma membrane and are released by exocytosis.

The specific biogenesis of EVs ensures an enrichment of molecules compared to the cell of origin, which produces a distinct molecular signature and EVs are assumed to play a significant role in cell-to-cell communication with mechanisms yet to be fully understood[102].

Over the last decade, research on EVs and especially exosomes as a potential biomarker for various diseases have grown[103], yet, the role of plasma EVs as a potential biomarker for early OA are sparse[104-107]. Studies to date have not investigated all possible miRNAs and their results are mainly inconsistent. To the best of our knowledge, reports on identification of all

possible miRNAs in plasma EVs in cartilage pathologies are lacking and knowledge on distribution of canonical miRNAs and isomiRs have not previously been explored, as is the case for the distribution of 5p and 3p strands and their related isomiRs.

Research gap

The main research gaps addressed in this thesis are the long term costs following knee cartilage surgery and epidemiological data on compensation claims following these procedures. We also investigate a potential circulating biomarker for OA.

Thesis aims

Overall aims

The overall aim of this PhD-thesis was to increase knowledge on surgical aspects for the treatment of FCDs in the knee joint and to evaluate circulating miRNAs as a potential biomarker for early OA.

Specific aims

Paper	Specific aims
I	To investigate differences in long term costs at 5 years following MF and ACI for surgically treated FCDs in the distal femur based on preexisting high level studies.
II	To evaluate compensation claims following FCD surgery in Scandinavia from 2010 to 2015 and identify potential areas of improvement.
III	To compare differences in plasma EVs miRNA expression levels between 23 patients with OA and 23 controls.
IV	To identify the content of miRNAs and isomiRs in plasma EVs and evaluate differences in the distribution of 5p and 3p strands in 46 persons.

Summary of the papers

Paper I

A meta-analysis was performed based on 4 pre-existing level 1 and level 2 studies published in the PubMed database comparing PROMs following MF and ACI for FCDs in the distal femur with minimum 5 years follow-up. 170 patients underwent MF and 149 ACI. Decision trees were constructed and costs following MF and ACI were calculated and compared. MF had substantially lower total costs at 5 years (€5150) compared to ACI (€14,491). The main cause was related to the primary surgery, where ACI was far more expensive than MF (€11,013 and €3254 respectively). Costs related to cell culturing, longer hospitalization, more physiotherapy and that ACI are performed as a two-stage procedure were the main contributors for higher costs following ACI than for MF related to the primary surgery. In fact, MF had slightly higher total costs connected to revision surgery (€821) than ACI (€703). Both MF and ACI had statistically clinical improvement in the weighted average of PROMs at 5 years compared to baseline. The costs of a 1-point increase in the reported PROMs from baseline to 5 years follow-up were lower for all PROMs following MF than after ACI. A sensitivity analysis demonstrated that a 66% reduction in total costs at 5 years following ACI equaled the total costs following MF at 5 years. If costs for hospitalization, physiotherapy and sick leave was identical for MF and ACI, a 41% decrease in costs after ACI would yield identical total costs related to the primary surgery.

Paper II

An observational cross-sectional study was performed to obtain and evaluate compensation claims following FCD surgery in Scandinavia from 2010 to 2015. We only identified 103 compensation claims during the study period, 43 (42%) following AD, 54 (52%) after MF, 3 (3%) following MP and 3 (3%) after ACI. Of the 103 claims, 36 (35%) were granted compensation, mainly following AD (21, 58%) and MF (13 (36%). Inadequate surgical technique and hospital-acquired infection were the leading causes for granted compensation, where 89% of the compensation claims due to infection was granted. Pain was a common reason for filing a compensation claim, but only a minority (14%) was granted compensation. The results demonstrate that compensation claims following FCD surgery are rare and suggest a lack of information to patients on compensation claims from health care personnel.

Paper III

A case control study was conducted to compare differences in plasma EVs miRNA expression levels between OA patients and controls using next generation sequencing (NGS) technique. 23 pairs of patients with OA and controls matched by age, sex and body mass index were recruited from the Musculoskeletal pain in Ullensaker Study (MUST)[108]. The groups only differed in the distribution of clinical and radiographic OA in hips and knees. Plasma EV isolation and miRNA sequencing were performed by Qiagen Services (Qiagen, Vedbaek, Denmark). Filtering on sequencing depth normalized values was applied and a threshold of 5 reads in the lowest sample was used as a detection limit. We identified a total of 177 canonical miRNAs in plasma EVs. However, we did not detect any significant differences in plasma EV expression levels between the 2 groups. In fact, 12 miRNAs associated with OA and the 20 most abundant miRNAs detected in our study showed very similar plasma EV expression levels between patients and controls. Our results indicate that this is a tightly controlled process, but we were unable to identify any plasma EVs miRNA that can potentially act as a biomarker for early OA.

Paper IV

In paper IV, the aim was to identify the content of miRNAs and isomiRs in plasma EVs and evaluate differences in the distribution of 5p and 3p strands across 46 individuals. The study population is the same as in paper III. In paper III, the 23 pairs of patients and controls were treated as 2 groups. As paper III did not reveal any differences in plasma EV expression levels between the 2 groups, paper IV analyzed all canonical miRNAs and isomiRs found in plasma EVs from the 46 individuals as one group. The same filtering and detection limit described in paper III was used for paper IV. 177 canonical miRNAs and 1716 isomiRs were detected, where two-thirds of the detected miRNAs had isomiRs. In 52% of the cases, isomiRs were found to be more abundant than the corresponding canonical miRNA. In regard to the distribution of 5p and 3p strands, both strands were detected in 32% of the canonical miRNA sequences. Only 5p were found in 36% and only 3p in 29% of the sequences. As noted in paper III, our findings suggest a tight control of synthesis and secretion into EVs. As isomiRs were found so abundantly, they could be considered for future biomarker analysis.

Methodological and statistical considerations

Medical research is original research that is conveyed with scientific methodology to obtain new knowledge, where the study method determines the informativeness and value of the research. Ensuring proper planning and choosing the most suited study method before conducting a medical study is of outmost importance, thereby securing value and reducing bias of the study. The study method to be chosen is dependent on the research question, but also what is possible to perform given the available resources, personnel and timeframe. Broadly speaking, medical research can be divided into primary and secondary research[109]. Primary research is be divided in epidemiological research, clinical research and basic/experimental research. Secondary research summarizes available studies and includes systematic reviews and meta-analysis. In the following, papers I – II and papers III – IV will be discussed separately.

Papers I – II

A systematic review refers to the whole research process from planning to completion and aims to answer a specific research question using pre-defined criteria. Reviews are at the top of the knowledge pyramid in evidence-based medicine[110], but require adequate amount of data to draw conclusions. A meta-analysis refers to a group of statistical techniques that expedite data from multiple studies to be combined and analyzed as an original dataset[111]. A meta-analysis does not involve more than putting the numerical data together and is different from a systematic review as the latter is a detailed review that aims to reduce bias at all stages of the process and not only the numerical data. A meta-analysis increases the power of a study by enhancing the sample size, providing a more accurate effect estimate. A meta-analysis was conducted in paper I to obtain numerical data from the identified studies from the search in the PubMed database. As for all research methods, meta-analysis is associated with limitations. Especially, summarizing vast amount of research data from several studies to a single number is controversial[112]. If one aims to compare results that are too different to combine, performing a meta-analysis would not be sensible. Another limitation is that meta-analysis may summarize data from different study designs introducing selection bias. To reduce bias, we chose to include only pre-existing clinical studies with evidence level 1 and 2. As we aimed to compare costs in Euros following MF and ACI for FCDs on the distal femur, decision trees were constructed (Figure 11).

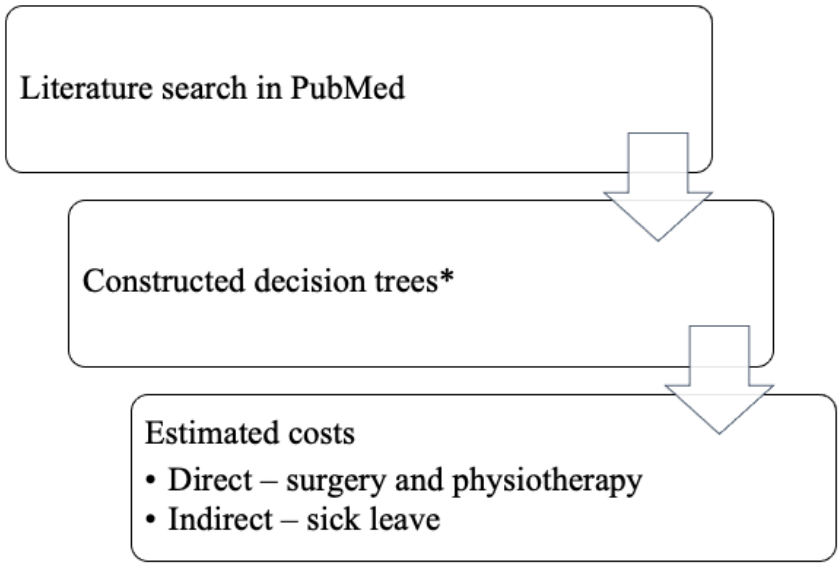

Figure 11. A schematic figure of the methods used in paper I. *Standard method for economic evaluation of health care program by Drummond et. al[59].

The constructed decision trees were based on the method for economic evaluation of health care programs described by Drummonds and collegues[59]. Other ways of constructing decision trees exists, but the method by Drummond et al. is one of the most used methods for economic evaluation and was deemed suitable for our study.

By combining the results in a meta-analysis, we increased the number of observations and hence increased the statistical power and improved the effect estimates. One way to assess the impact of various selection criteria on the results when combining data from different studies is to perform a sensitivity analysis. A sensitivity analysis is a method to test the robustness of the findings to various assumptions[113]. This what-if-or simulation analysis aims to determine the effect of various inputs variables on the target variable. We analyzed primary direct costs and total costs at 5 years following MF and ACI, and also performed a sensitivity analysis if certain costs were identical for MF and AC. At present, ACI is performed as a two-stage procedure. If we assumed that ACI could be performed as a one-stage procedure, a new sensitivity analysis should be performed given these assumptions. Almost certainly such an analysis would reveal lower costs following ACI but were not performed in the present study.

An optimal CEA of MF and ACI should have been based on a large, multinational randomized clinical trial (RCT) or a prospective cohort study comparing MF and ACI with non-operative treatment with minimum 5 – 10 years results. A RCT or a prospective cohort study would gradually increase the study population as new cases are included, leading to a larger study population and more accurate statistics. In this way, a proper study population would be available and uncertainties on decision trees and unit prices could have been reduced yielding higher transferability of the results than our study. Initiating an international CEA is difficult to perform due to both institutional and financial and possibly legal restrictions. Hence, a large national RCT or a prospective cohort study would be a more realistic CEA to perform. However, both studies are time consuming and expensive to perform, with the risk of loss to follow-up. Most likely the timeframe would exceed several years, increasing the costs and likelihood of drop-outs.

A cross sectional study design is a study design under epidemiological research[109]. This method enables obtaining data of a large sample size at a single point in time, even though recruitment may take place over a longer time period. A cross sectional design was employed in Paper II (Figure 12).

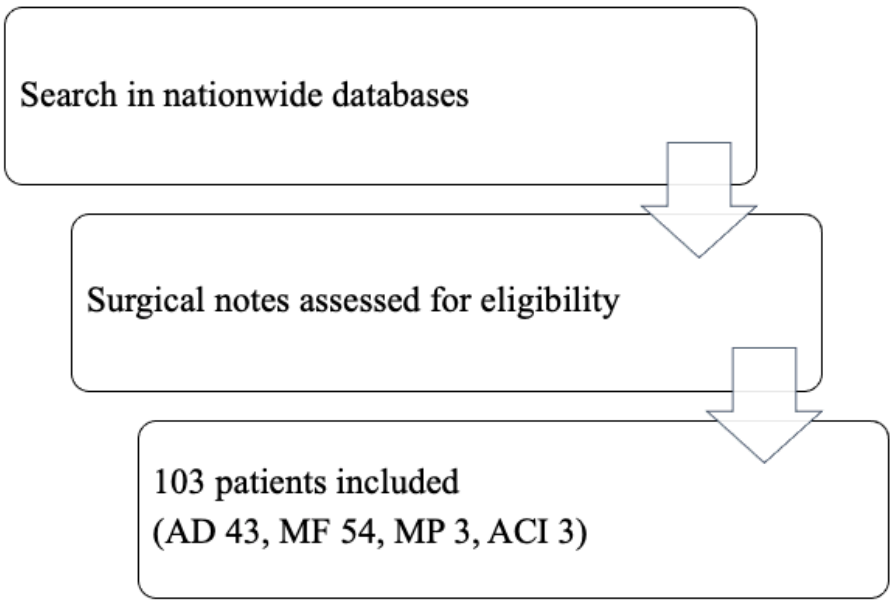

Figure 12. A schematic figure of the methods used in paper II.

A cross sectional register study offers several advantages as this is easy and quick to perform and are not associated with huge costs as other study designs. Another strength with a cross

sectional study is that there will be no loss to follow-up, as participants are only subject to one evaluation. A cross sectional study design can offer important knowledge on prevalence and incidence of a disease in a population if the data is reported in agreement with the STROBE guidelines[114]. This could be useful when allocating resources and planning health care services[115]. Indeed, a report from Cochrane revealed that cross-sectional studies yield comparable results than similarly conducted RCTs[116].

Estimating a prevalence of compensation claims following knee cartilage surgery enables a direct comparison with compensation claims following other conditions. Randsborg et. al reported 24 compensation claims yearly in Norway following anterior cruciate ligament[84], higher than our finding of 17 claims per year for all three countries. Additionally, knowledge on prevalence and incidence can educate health care personnel about the risks of treatment errors. One advantage of a register-based cross sectional study is that it is not prone to non-response bias such as survey-based cross sectional studies. By obtaining information directly from the registries, this bias can be avoided. Other strengths with a cross sectional study design are the possibility of collecting information for rare diseases, allows for subgroup analysis and can generate new hypothesis for further investigation.

However, using a register for data collection are associated with some challenges. For instance, if patients have suffered a treatment error, but not filed a complaint, this may elicit inclusion bias. Another example of a potential bias in cross sectional register study is that some cases may have been mislabeled in the register leading to selection bias. We screened the databases using a wide range of predefined diagnosis and procedure codes, but if patients were mislabeled, they would not have been included. Another drawback with this study design is that a cross sectional study will not explain causation, nor will it be able to assess whether new cases have developed, as this necessitates a longitudinal assessment. Albeit this could be avoided if cross sectional studies are repeated at different times to assess trends over time. But then again, caution is warranted as different participants are included at different time points. Another limitation of this study design is that is does not allow for a control group which can be useful in interpretation of the results. Using registries for analysis can also have limitations in factors that cannot be investigated. In our study, the registries did not contain demographic information such as ethnicity, socioeconomic status and insurance status. These factors could serve as subgroup analysis but were not available and subgroup analysis could therefore not be performed.

An alternative study type to identify treatment error following knee cartilage surgery is a prospective cohort study or a RCT. Such study designs would offer benefits over a cross sectional study as they should include a control group which could differentiate the findings on treatment errors and report new cases as the assessments is performed more than once. However, as mentioned above, these study designs are associated with high costs, are time consuming and requires additional personnel. Besides, since compensation claims are so rare, it will be practically impossible to include enough people in a prospective study.

The statistical analysis in cross sectional studies depends on the design. Usually, the statistical analysis is similar to that of case control studies using logistic regression and calculating odds ratios. One major limitation of paper II was the unknown incidence of cartilage surgery in Scandinavia. This implicates that a power analysis or a post hoc analysis was deemed impossible and the ability to detect any effect is uncertain. If the incidence was available, comparing nominal variables for more than two groups would have been performed using either independent t-test or the Chi-square test. Another effect of performing a power analysis, is to avoid type II errors. Lastly, due to the lacking incidence of cartilage surgery, the statistics used in this paper are mainly descriptive.

Papers III – IV

Paper III aimed to compare differences in miRNA plasma EV expression levels between 23 patients with OA and 23 controls. To answer this, we chose to conduct a case control study. A case control study was chosen as study design as a case control study compares patients with OA (cases) with patients without OA (controls) and retrospectively compares the frequency of risk factor (EVs miRNA) present in each group to determine a relationship between EVs miRNA and OA.

This study design is observational, as there is no intervention or attempt to alter the course of OA (the studied disease in our case), with a main goal to estimate odds between the 2 groups. A case control study is particularly useful when the disease or outcome is rare, when the disease or outcome has a long induction or latent period, when exposure data is expensive or difficult to obtain or when little is known about the risk factor. The latter enables testing of associations with multiple potential risk factors, which was the case for our study. The testing of associations with multiple potential risk factors are also the case for cohort studies.

However, such a study design is much more time-consuming and costly than a case control study as previously mentioned.

One disadvantage with a retrospective study design such as a case control study is the potential of recall bias. Studies may rely on patient's memory or on patients with a condition that are more motivated to recall risk factors than the controls without the disease. Another potential disadvantage of a case control study is the selection of cases and controls. To identify cases, a suitable case definition should exist. To avoid inclusion bias, care should be taken in the way cases are selected. These factors, confounders, can significantly affect the result and must always be carefully considered in patient enrollment and in statistical analysis. It can be difficult to find a suitable control group for a case control study. The risk factors and confounders should reflect the general population "at risk" of becoming cases. Our study population was extracted from the MUST study[108]. To reduce factors that could influence the results, pairs were matched by age, sex and body mass index. 23 pairs were deemed adequate as sample size. However, there was no calculation of the sample size, as the use of NGS on plasma EVs as a biomarker for OA has never been published earlier.

Experimental research, the last of the subgroups within primary research, differ somewhat from clinical and epidemiological research[109]. In paper IV, the aim was to identify the content of miRNAs and isomiRs in plasma EVs and evaluate differences in the distribution of 5p and 3p strands. As paper III did not detect differences in plasma EV miRNA expression levels, the two groups were in paper IV treated as one group of 46 individuals. Hence, the study can be regarded as an experimental research study. Laboratory studies are associated with a tight control of variables and enables use of complex equipment, whereas disadvantages can be related to the realism of the project and may be subject to experimenter bias, meaning bias that arise from expectations of the study that affect behavior.

When performing laboratory studies, securing adequate external and internal validity are of paramount importance. External validity refers to the generalizability of the study to real life and can be challenging as the experimenter controls the variables. To increase internal validity, standardized experimental conditions are required.

The methodological steps leading to data analysis were identical for papers III – IV and are described in detail in the method section of the papers (Figure 13). In the following,

additional considerations of the methodological steps are presented as they all can affect internal validity and hence, our findings.

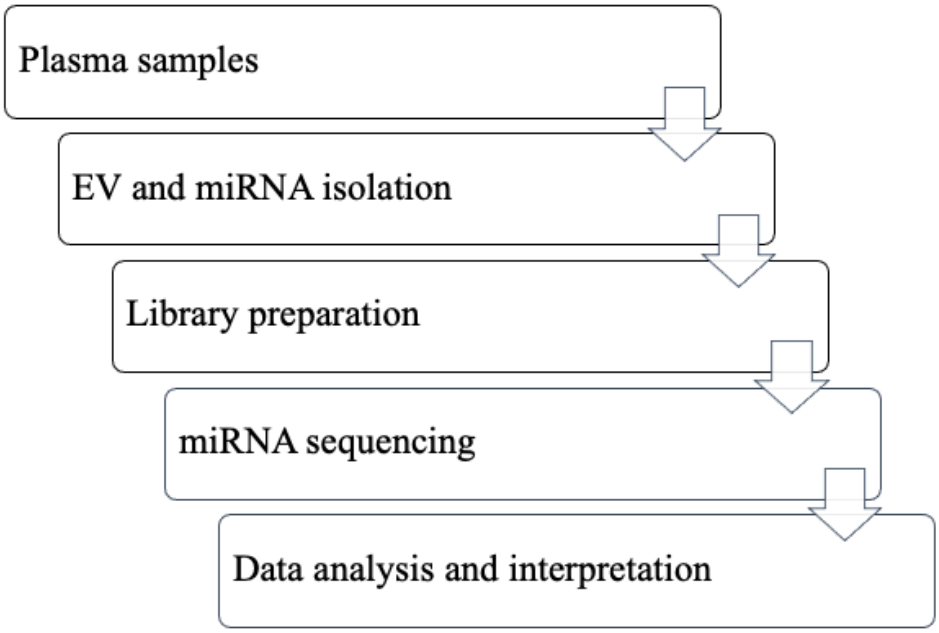

Figure 13. A schematic figure of the methods used in papers III – IV.
Quality control were performed at steps 2 and 3.

Plasma samples

The preferred way to handle and store plasma have been discussed with conflicting reports[117,118]. Proper biobanking of blood samples are a cornerstone to progress in translational research in medicine[119]. Any variations in collection, centrifugation and handling of the plasma samples could affect findings. The blood samples used in our analysis were centrifugated within 30 minutes after blood sampling. In regard to storage temperature of plasma samples, – 80° is most often recommended, although some claim higher temperatures might be sufficient[120]. The description of storage time of blood has been limited. Our plasma samples were analyzed not later than 7 years after collection. It has been demonstrated that storage time explains up to one-third of plasma protein concentration variation in frozen samples for samples stored for up to 30 years and storage time should be included as a covariate[121]. We did not include store time as a variate in our analysis and hence, this could affect the results. However, by comparing pairs adjusted for age, sex and body mass index, the effect on our results is assumed to be minor.

EV and miRNA isolation

There is a broad range of techniques available for isolating EVs[100,122]. From density centrifugation, affinity capture and membrane filtration, they all have different characteristics in yield, purity and scability[122,123]. However, these studies usually involve material harvested from cell culture media and not blood. Isolation of EVs from whole blood and plasma adds further challenges due to the high-abundance of circulating proteins and lipoprotein particles and the limited availability of valuable specimens[122]. The gold standard for isolation of EVs up until recently has been differential ultracentrifugation[124]. However, this technique is time consuming, expensive and subject to variability and lately, several commercial kits have become available[124]. It has been reported that the exoRNeasy Serum Plasma Kit (Qiagen, Vedbeak, Denmark) can capture almost all mRNA from plasma and is equal or even better than the former in mRNA yield[124]. Indeed, reports state this kit has superior isolation characteristics compared to ultracentrifugation and other isolation kits[124,125].

EV size measurement

The Zetasizer Nano ZS system (Malvern Panalytical, Malvern, UK) was used according to the manual from the manufacturer to measure EVs size.

Western blot (immunoblot)

Western blot (WB) is a method widely used for detection of proteins in tissue samples, blood, cell lysates or cell culture supernatants utilizing antibodies. The identification and quantification of plasma EVs using WB are well-described in the literature[126]. To identify EVs, we used rabbit anti-human ALG-2-interacting protein X (ALIX), rabbit anti-human tumor susceptibility gene 101 (TSG101) and rabbit anti-human CD81 (Abcam, Cambridge, UK) as primary antibodies and horseradish peroxidase-conjugated horse anti-rabbit IgG (H + L) as secondary antibody (Vector labs, Burlingame, CA). ALIX and TSG101 are proteins involved in sorting of cargo into EVs whereas CD81 are predominantly found on the surface of EVs[126,127]. By combining these 3 EV markers and based on our identification of these antibodies in our studies, we believe the identification of EVs are obtained.

Reverse transcription-quantitative real-time polymerase chain reaction (RT-qPCR) and next-generation sequencing (NGS)

RT-qPCR is established as a powerful method and one of the most used techniques for quantification of RNA due to the high sensitivity, specificity and reproducibility of this

method. RT-qPCR is divided in 4 steps[128]. First, preparation of RNA and second, reverse transcription (RT) to cDNA. The third step involves PCR amplification of cDNA before the final step is data analysis. cDNA template is doubled in the first PCR cycles, leading to exponential amplification. In this phase, the formed PCR product is proportional to the available amount of RNA in the starting material. Thereafter, the reaction slows down, and the PCR product is not doubled mainly due to limitations of the reagent[129]. The amount of PCR product formed in the exponential phase is detected with RT-qPCR recording the signal intensity caused by fluorescent dyes and probes that bind to the PCR product formed following each cycle of PCR. RT-qPCR then determines the baseline amount of target RNA. Quantification of miRNA can be performed in two ways. Absolute quantification (AQ) determines the exact numbers of initial target RNA molecules by comparison with standard curves, whereas relative quantification (RQ) calculates the change in expression relative to a reference sample without listing the exact number of target RNA molecules[129].

Two drawbacks with this technique are that RT-qPCR can only detect known sequences and RT-qPCR normally do not differentiate between highly similar sequences, with the consequence that isomiRs are most likely misinterpreted as canonical miRNAs. In a submitted but yet unpublished paper, it has been demonstrated that the isomiRs of miR-140-3p were detected with the probe for the canonical miRNA, highlighting this issue. In paper III – IV we therefore opted to use next-generation sequencing technique (NGS)[130].

NGS follows miRNA to cDNA conversion and PCR to library preparation. NGS offer the same high sensitivity, specificity and reproducibility as RT-qPCR, but also enables detection of unknown sequences and hence, has a higher sensitivity to quantify rare sequence variants and higher power to detect novel genes[131]. NGS offers an advantage over RT-qPCR when assessing many targets as NGS can identify variants across thousands of target regions making it an ideal technique when searching for a potential biomarker for OA[132]. Another important feature of NGS is that it provides more data from smaller RNA amounts, also an essential treat when searching for a biomarker[133].

NGS is based on massively parallel sequencing technique where the sample DNA is fragmented into smaller pieces and linked to adapters to generate the library which is used as templates for the synthesis of DNA fragments. Each of the nucleotides (nt) in these fragments

are marked with a fluorescent probe ensuring identification of the sequences. The obtained sequences are then compared with the human genome reference sequence.
However, NGS requires a dedicated data-handling workflow and are prone to error if not sequenced multiples times. Some therefore advice a combination of different methods for the verification and accuracy of the results.

Our results demonstrated a small yield of purified plasma EVs (less than 0.1 μg of total RNA in 1 mL of plasma) in 500 μL of plasma. NGS usually requires more than 1 μg of total RNA, this could cause variability in our findings. However, as the numbers of miRNAs identified was similar to other reports and the spike-in sequences were found at reproducible levels[107], this lends support to our belief that our findings truly represent the relative concentration of miRNAs and isomiRs in EVs.

Identification of isomiRs

In paper IV, isomiRs were detected individually for each count variable of the identified miRNAs. Reads were mapped to known miRNAs according to the miRNA database and investigated for the presence of different isomiRs by changes in start or stop position, or mutations within the read. Identification of isomiRs was performed by Qiagen Services (Qiagen, Vedbaek, Denmark).

The Minimum Information about a Microarray Experiment

The Minimum Information about a Microarray Experiment (MIAME) checklist is touted as a guideline for conducting microarray analysis[134]. The MIAME checklist ensures a standard for recording and reporting microarray-based gene expression data. MIAME was used as guideline for papers III – IV, ensuring methodologic validity of our studies.

Statistics in papers III – IV

Statistical analysis in case control studies usually involves calculating odds ratios and almost all studies can be subject to sample size calculation. Papers III – IV involves an early exploratory study design where sparse data were available to perform power calculation. In fact, the NGS technique used in these papers for the detection of plasma EVs miRNA and isomiR in OA are not previously described, and consequently, power calculations were not performed. To make allowance for potential confounding factors, statistical adjustments is made. Pearce have claimed that there are two common misconceptions about case control

studies[135]. First, that matching itself eliminates controls confounding by the matching factors and if matching has been performed, a matched analysis is required[135]. In paper III, cases and controls were paired based on age, sex and body mass index. According to Pearce, the matching process can make the controls more similar to the cases for the exposure as well as the matching factors[135]. As we aimed to compare differences in plasma EV expression levels of miRNAs between cases and controls, we recognized matching a necessity.

The difference in the expression level values for each pair of case and controls were calculated and a paired t-test on whether the average difference is zero were performed. Using this method, 88 miRNAs and isomiRs with significance was identified ($p < 0.05$). However, as we did multiple testing, the limit for significance was adjusted first using the Holm-Bonferroni procedure and then using the Benjamini-Hochberg procedure. These methods thus calculate a significance limit for each of the cases, and the result was considered significant if the p-value from the t-test is lower than the current limit. Following adjustment using either the Holm-Bonferroni or the Benjamini-Hochberg procedures, no miRNAs or isomiRs were statistically significant between the two groups.

Ethical considerations

Broadly speaking, all medical treatment can be considered an experiment. Since the famous words “First, do no harm” by Hippocrates[69], ethics has gradually gained a vital position in medicine. However, it was the discovery of the horrendous experiments conducted by the Nazi doctors on people in the concentration camps which led to ethical codes being formalized to prevent recurrence. The four fundamental principles of ethics are autonomy, non-maleficence, justice and beneficence[136]. Since the establishment of the Nuremberg codes after World War II and the World Medical Association recommendation to establish independent committees of research ethics in 1975, medicine has taken giant steps in securing patients' individual rights and informed consent according to these four ethical pillars.

In Norway, research on anonymous data and material does not require informed consent or ethical approval from The Norwegian ethics committee as stated in the Health Research Act. Nonetheless, the importance of informed consent should not be taken lightly. Prior to inclusion in clinical trials, all participants should be given correct information about the study and time to consider the potential benefits and risks of participating in a study. Paper I raise an interesting ethical dilemma. As meta-analysis uses anonymized data, should inclusion criteria in meta-analysis include a written statement that all included studies are approved by an ethical committee?

Patient autonomy should never give way to the researcher's desire for the patient to participate in a study. The necessity of registering clinical trials in databases such as clinicaltrials.gov are also an important element of ethics as this increases transparency and strengthens research and makes it reproducible. Another significant constituent is the guidelines launched by The International Council for Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use[137]. Primarily guided towards pharmaceutical research, the Good Clinical Practice serves as an international ethical and scientific quality standard in all phases of trials involving human subjects[138].

The increased focus on molecular medicine in research has launched complex ethical discussions and guidelines concerning biobanking activities[139,140]. Biobanking are now an integral part of research and healthcare and includes activities from collection, storage and sharing of research material. The Declaration of Helsinki is a statement of ethical principles

for medical research involving humans[141]. Despite its pivotal role, this declaration only summons the need for research ethics review and informed consent[142]. Collected material are supposed to be stored de-identified, and inaccessible for others except the listed personnel named in the study protocol. This was also the case for papers III – IV, where the blood samples were stored de-identified at an approved biobank at Oslo University Hospital, Norway and were inaccessible for others except for the principal investigator of the MUST study[108]. This implies that biobanking is low-risk and de-identifiable samples provides satisfactory privacy. However, stored blood samples may be identifiable through genetic analysis and hence, questions whether storage of de-identifiable blood samples secures adequate privacy have been raised. Ashcroft et. al commented that if material is collected without disclosure, understanding and informed consent, biobanking is not as low risk as it initially appears[142].

Sharing of research material have had profound effect on health outcome during disease outbreaks, for example during the outbreak of Ebola virus in 2014. The biobank in Sierra Leone contained large amounts of samples which was shared between countries. Ethical questions concerning the absence of a complete inventory of the samples collected, the consent for future use and the ethical adequacy of the agreements between the countries have been raised[142]. During the ongoing outbreak of the coronavirus in 2020, these ethical concerns are most likely still valid.

Before enrollment in the MUST study[108], participants signed a written consent that the collected blood samples would be subject to further research in the future and the study was approved the regional ethics committee. The use of previously collected information or material for future research raises ethical considerations. For instance, participants may not know what kind of future analysis will be performed and for what purpose. In a cross-sectional survey, Abou-Zeid and colleagues reported that 66% of adult Egyptian patients would donate their samples for future research[143]. However, many favored a consent model that included an option restricting the future research to the illness being studied. In a population-based risk factor study from Sweden, participants were contacted 11 years later and asked for informed consent to participate in genetic studies[144]. Stegmayr found that 93% gave their consent provided an ethics committee had approved the research, albeit 22% wanted to be informed and give their consent to every new specific project. This highlights

the importance of ethics committee reviews, adequate information and obtaining informed consent before enrollment occurs.

Power analysis is often used to determine sample size and can serve an example of non-maleficence. A power calculation is important, as this estimates the required number of patients to avoid type I and type II error. Power and sample size estimations are influenced by several factors, some controllable, other not[145]. The use of too many participants is without doubt unethical, as this wastes time, money and effort, in addition to subject participants to potential harm. It may be relatively easy to predict physical harm, i.e., the discomfort when giving a blood sample. However, other forms of harms may be difficult to detect. No research is without risk, but research with possible permanent damage to participants should not be permitted. Alongside the researcher's evaluation of potential side-effects of research, ethics committees are invaluable in considering possible risks to participants and oversee that researchers minimize the possible harm.

Non-maleficence often goes hand-in-hand with beneficence, the antonym of non-maleficence. Beneficence is touted as the moral ideal of research, to maximize the benefits to an individual or to society. The WHO Declaration of Alma-Ata from 1978 states that "people have the right and duty to participate individually and collectively in the planning and implementation of their health care"[146]. Such participation from the patients (users) may contribute to more relevance of the research with different perspectives enhancing beneficence. This has been shown by Tallon et al, where mismatches in research priorities regarding OA of the knee have been reported[147]. They reported that patient priority especially focused on knee replacement and education and advice, whereas the majority of trials done focused on drugs[147]. This should serve as a reminder to all parties involved in research that patients can certainly contribute to research and not just be a passive part of it.

Discussion

Paper I

Based on level 1 and level 2 studies, we found that MF was associated with substantially lower costs at 5 years compared to ACI for the surgical treatment of FCDs in the distal femur. The main cause was related to the primary surgery, where MF was far less expensive than ACI. These findings are consistent with a systematic review by Schrock et al. which stated that MF was less expensive than MP and ACI for the treatment of FCDs in the knee[65]. Similar to our findings, Schrock and associates found that the costs of a 1-point increase in reported PROMs was lower following MF compared to the other surgical treatment options[65].

Hospitalization was one important factor that yielded higher costs following ACI. We assumed that ACI required 3 days of hospitalization, although the exact length of hospitals stays vary among patients. Contrary to our assumptions, Mistry et al. assumed that patients treated with ACI were outpatients and MF were inpatients, and thus reported that ACI was more cost-effective than MF[66]. However, these assumptions are unlikely to reflect real life scenario. The National Institute for Health and Care Excellence (NICE) founded the review by Mistry and colleagues which included six articles[66], all with shortage of long term results and good quality of life data were lacking. They defined long term as at least five years but did not restrict study designs. Notably, they found that only a minor portion (12.5%) required revision surgery, which is substantially lower than our findings and do not comply with the 14 – 15 years results from a level 1 clinical trial comparing MF and ACI[11]. Another difference from our study is that Mistry et. al used quality adjusted life years (QALY) as a comparative measure when evaluating cost-effectiveness[66]. QALY is a multiplicative from the patient's health related QoL and the length of time, integrating the impact on both quantity and quality of life and is presented on a scale between 0 (death) and 1 (perfect health)[148]. Although QALY is routinely used to assess cost-effectiveness, it is prone to variability, especially related to demographic belonging[149], which is most likely the case in the review by Mistry et. al[66].

Costs related to cell culturing were an important factor that yielded higher total costs for ACI. The costs due to cell culturing most likely differ among laboratories and among countries. We used a fixed price at €4050 for cell culturing expenses, whereas Mistry et. al used an

implantation cost of £2396 (equaling €2654) at the request of NICE[66]. If costs related to cell culturing and implantation were reduced, costs following ACI would subsequently be lower. This is highlighted in a study by de Windt et al. who compared MF, ACI and a single-stage tissue engineering procedure[68]. They reported that MF was the most cost-effective treatment options for smaller defects, whereas a single-stage tissue engineering procedure could be recommended for larger FCDs instead of ACI.

The lesion size in our study differed slightly between the two groups (2.5 cm^2 for MF and 3.2 cm^2 for ACI) but were assumed not to affect our results. MF are usually not indicated for larger defects due to deteriorating clinical effect and increased failure rates over time[11,150]. It is unclear if MF are cost-effective for larger defects. Everhart et. al have recently published a systematic review of 22 studies comparing cost-efficacy between MF, MP, ACI and MACI[151]. Like us, they found that MF are cost-effective in treating small FCDs but was not found cost-effective for FCDs larger than 3 cm^2. However, they did not account for indirect costs due to sick leave and productivity loss and therefore results should be interpreted with some caution[151].

Technical advances in cartilage surgery such as nanofracture, scaffolds and matrix-assisted ACI (MACI) are gaining popularity. Albeit long term studies are lacking, some interesting findings have been published on short term follow-up. Frappler et. al have compared costs of MF with a BST-CarGel adjunct to MF and reported that the former could potentially reduce costs compared to MF alone[152]. Kim et al. compared MF with a collagen-augmented chondrogenesis technique for the treatment of FCDs in the knee[153]. They found that the collagen-augmented technique resulted in better cartilage filling of the defect. However, these results should be interpreted with caution. The majority of the study population was over 50 years old, and approximately 80% had radiological OA K-L grade 2 or higher and half of the participants underwent concomitant high tibial osteotomy, all factors that might influence outcomes. MACI have been compared with MF in clinical studies, and the results seem to be superior to that of MF in regard to PROMs and adverse events[154-156]. Brittberg and colleagues recently published 5 years results based on a clinical trial comparing MACI with MF for FCDs[157]. They found improved clinical results following MACI compared with MF for lesions larger than 3 cm^2, but no difference was found in regard to the MRI evaluated cartilage filling of the defect[157].

Niemeyer performed a CEA of MF and MACI based on 127 patients from the German statutory health insurance[158]. Similar to our findings, MF was associated with lower mean total costs at 5 years and marginally higher revision costs than ACI[158].

Lastly, mesenchymal stem cells have only recently been introduced as a treatment option for FCDs and OA[159]. Although comparative results are yet to the published, one study aims to evaluate the cost-effectiveness of a one-stage cartilage repair using mesenchymal cells and ACI compared to nonsurgical treatment for focal articular cartilage lesions of the knee[160]. Results from ongoing clinical trials comparing mesenchymal stem cells with other cartilage procedures are expected in the next few years, and these studies should also be subject to health economics analysis.

No study has compared cartilage procedures with AD or non-surgical treatment options, however, there are studies enrolling participants at the moment[52,160,161]. High level clinical studies with follow-up exceeding five years are necessitated to better determine the optimal treatment of FCDs. Such studies should also include a CEA, that will inevitably supplement the clinical decision process.

Paper II

To the best of our knowledge, this cross-sectional study is the first to illuminate compensation claims following knee cartilage surgery. During the study period from 2010 to 2015, only 103 compensation claims following knee cartilage surgery were identified when searching the Norwegian, Danish and Swedish databases. 36 (35%) of the 103 claims were accepted. The acceptance rate of one-third is similar to other studies on compensation claims following orthopedic surgery[84,162].

The majority of claims were after AD and MF, accounting for 94% of the claims. Of the 36 accepted claims, AD accounted for 21 (58%), MF for 13 (36%), MP for 1 (3%) and ACI 1 (3%). Clearly, AD and MF are more often performed than MP and ACI as they are relatively simple and low-cost procedures. This explains the dominance of AD and MF in regard to compensation claims compared to MP and ACI. The low occurrence of compensation claims following MP and ACI are in line with earlier studies reporting that major adverse events following these procedures are rare[163,164]. However, there is a difference between

compensation claims due to treatment error and the incidence of complications. We have thought that some patients that undergo MP and especially ACI have tried other surgical treatment options that have failed, are carefully selected and well informed patients that could affect the number of compensation claims following MP and ACI.

The leading causes for accepted claims were inadequate surgical technique (no further explanation was given), hospital-acquired infection, nerve injury and delayed diagnosis and treatment. Notably, 89% of the compensation claims due to infection was accepted, all following AD. Infection is a potential serious adverse event following knee cartilage surgery that may elicit increased morbidity and mortality with the potential of huge health care costs. Several of the accepted claims due to hospital-acquired infection was related to the exception clause. This clause leads to approval of compensation claims even if no treatment error has occurred and is a pragmatic policy decided by the Scandinavian compensation systems. However, not all compensation claims due to infection will automatically be accepted as all claims are evaluated independently. Patients with poor compliance and increased risk of infection may not be granted compensation even if infection occur.
Pain was a common reason for filing a compensation claim, but only a minority (14%) was granted compensation. This is accordance with other studies illustrating that pain does not serve as a cause of compensation[82-84].

To date, knowledge on compensation claims following knee arthroscopy is sparse at best and absent for knee cartilage surgery as far as we know. One study has reported malpractice lawsuits following arthroscopic surgery in the United States over a period of 29 years[82], whereas one study from England and Wales evaluated litigation claims after knee arthroscopy over 15 years[83]. Shah et al. identified 162 claims following knee arthroscopy, unfortunately they did not specify which treatment given[82]. Similar to our findings, Shah and associates found that approximately two-thirds of the claims were rejected, and that infection was one of the three leading causes for compensation[82]. Different from our results, musculoskeletal complaint (stiffness, chronic pain and unsatisfactory result) and deep vein thrombosis were among the top three reasons for accepted compensation claims. The study by Harrison et al. identified 217 settled compensation claims, of which 58% resulted in compensation[83]. Anterior cruciate ligament (ACL) reconstruction accounted for one-third of all compensation claims in the study, but they did not report findings following knee cartilage surgery. The most common reasons for compensation were ACL failure, infection, nerve injury and

retained metal. It is worth mentioning that the cases with infection presented by Harrison et al. was not awarded compensation due to this recognized complication (infection), but rather lack of early diagnosis or inadequate follow-up.

A study on compensation claims aim to improve patient safety. According to Tilma et. al, nationwide compensation databases hold valuable material on treatment errors and act as a source of designing efficient preventive initiatives yielding better patient safety[165]. This has recently been highlighted in a study from Sweden that stated that approximately 50% of the adverse events following total hip arthroplasty could have been prevented[166]. Our study identified several cases of treatment errors that are avoidable with adjustments of all phases of treatment from preoperative planning, positioning of the patient, adequate surgical technique and satisfactory postoperative management. Delayed diagnosis and treatment have in recent years been recognized as avoidable causes for compensation[84,167]. The implementation of safe surgery protocols launched by the World Health Organization is another important step toward reducing avoidable treatment errors[168]. Positively, we did not detect casualties or wrong sided surgery as was the case for the studies by Shah et al. and Harrison et al.[82,83].

Compensation is assumed to vary between countries and between the two main compensation principles, and this was obvious in our study. Among the 36 accepted claims, a total of €807,086 were paid in compensation with an average of €24,457. The average compensation is almost identical to the average compensation following treatment error after anterior cruciate ligament reconstruction in Norway[84]. According to the study by Harrison, the mean compensation in England and Wales was £47,330 (€52,214), whereas the average compensation is clearly different from the United States where Shah et al. found an average compensation of $848,331 (€733,486). This also demonstrates that treatment error following knee cartilage surgery carries a significant economic burden to society.

The number of compensation claims identified were remarkably low and most likely underreported. Denmark had in fact most compensation claims despite the fact that Sweden has twice as many inhabitants. This may be explained by cultural differences between the Scandinavian countries, rather than a difference in health care provision for this injury. It may indicate that Denmark has a better adopted system for compensation claims. In comparison, a study that evaluated compensation claims following anterior cruciate ligament reconstruction identified 24 annual compensation claims in Norway, mirroring the low numbers of claims

identified in our study[84]. Nonetheless, our study did indeed identify more annual compensation claims than the studies by Shah et al. and Harrison et al.[82,83].

The exact numbers of cartilage procedures performed in Scandinavia during the study period was unknown. Based on the reported incidence of knee cartilage surgery from Norway[9], at least 7500 annual cartilage procedures would be performed in Norway, Denmark and Sweden combined. Identifying only 17 compensation claims each year seems fairly low. The lack of incidence of non-surgically and surgically treated FCDs illuminates the need of nationwide knee FCDs registers similar to the arthroplasty registries[169]. Some countries are on the brink of establishing such registries, such as Germany[170]. Engen et. al have described the development of a pilot register for focal cartilage injuries in the knee, as well as mapping and reporting challenges[171]. They recommended that challenges concerning incomplete patient data and low registration compliance should be addressed before establishing a nationwide register. Such a register should include both conservative and surgically treated patients with PROM scores available and would offer several benefits including clinical research, improving treatment strategies and enhancing patient safety.

Paper III

Using the NGS technique, we identified 177 plasma EVs miRNA across 23 patients with OA and 23 matched controls without OA. Despite identifying a considerable number of different miRNAs, the expression levels of miRNAs in both groups were remarkable similar.

One of the most studied molecules as a potential biomarker for OA are serum oligomerix matrix protein (COMP)[172], which is found to be elevated in patients with hip and knee OA[173]. However, as for other potential biomarkers, including aggrecan, interleukins and urinary C-terminal telopeptides of type II collagen (uCTX-II), the results are so far inconclusive[89,172]. Beside from a diagnostic biomarker suggestive of early OA, a prognostic biomarker has been under thorough investigation the last decade. One report has demonstrated that the absence of aggrecanase-1 in synovial fluid was an indicator of ACI success[174]. This implies that future studies should focus on identifying biomarkers with diagnostic and prognostic characteristics.

Most studies on miRNAs as biomarker for OA are studies of animals, synovial fluid or chondrocytes. MiR-16-5p and miR-126-3p were found to be most abundant in our study and

both miRNAs are associated with OA. MiR-16-5p is reported to be higher expressed in OA cartilage compared to healthy cartilage and to target chondrocyte differentiation and homeostasis[97], whereas miR-126-3p is found to be lower in OA cartilage compared to normal cartilage[175]. Despite the association to OA, no differences in expression levels for either miR-16-5p or miR-126-3p were found between the two groups. This is also true for all of the other 175 miRNAs identified, as no difference in expression level was identified between persons with and without OA.

miR-140 has been extensively studied and launched as a potential biomarker for OA as this miRNA is assumed to be cartilage specific[176]. Miyaki et. al have demonstrated that deletion of miR-140 predisposed mice to OA[177], whereas studies on synovial fluid have reported that decreased expression levels of miR-140 is negatively related to OA severity[96,178]. Despite these interesting findings and that miR-140 was detected in our study, miR-140 in plasma EVs was not among the 20 most expressed miRNAs and no difference between the groups were found.

Despite the fact that miRNAs are largely found in the cell cytoplasm, miRNAs are also found extracellularly bound to proteins, lipoproteins or contained in vesicles[98]. Among circulating carrier proteins, AGO-2 is believed to be the most important. Arrayo et al. have demonstrated that a substantial proportion of circulating miRNAs are bound to AGO-2 and reported that these complexes were resistant to degradation[179]. Circulating miR-16-5p has previously been demonstrated to occur in plasma mainly attached to AGO-2[180]. As our results demonstrated high levels of miR-16-5p, we suspect some cross-contamination of AGO-2 is present in our EVs. This is further enhanced by Enderle et. al, which stated that currently available methods for detecting free circulating miRNAs are prone to contamination with AGO-bound miRNAs[124]. Both high-density lipoproteins (HDLs) and low-density lipoproteins (LDLs) are shown to be associated with miRNAs, but the latter only binds minor levels of miRNAs[99]. Circulating miRNAs bound to HDL- and LDL-proteins were assumed to not contaminate our findings.

Circulating miRNAs have recently gained attention as a potential biomarker for OA, where studies are mainly on serum or plasma. Beyer et al. found that let-7-e, miR-454 and miR-885-5p were predictive miRNAs for severe OA based on analyses of pooled serum samples from 13 patients scheduled for total hip or knee arthroplasty due to OA with 13 persons without

OA using a microarray screen[104]. Comparing serum miRNAs differential expression (DE) from 12 patients scheduled for total knee arthroplasty due to OA with 12 patients undergoing knee fracture repair surgery, Ntoumou and colleagues found a downregulation of miR-33b-3p, miR-140-3p and miR-671-3p in OA serum[106]. Conducting a microarray study comparing circulating miRNAs in plasma from patients with OA and controls without OA, Borgonio Cuadra et al. identified 12 elevated miRNAs in the OA plasma[105]. With a slightly different study design, Murata and associates compared 5 different miRNAs in plasma and synovial fluid in patients with OA, rheumatoid arthritis and health controls and found that miR-16 and miR-132 were lower in OA patients compared to controls[107]. The findings in these studies are divergent and the clinical efficacy are still doubtful and unproven. More recently, Rousseau et. al used NGS and compared miRNAs DE in serum in women with and without OA[181]. They found that circulating miR-146a-5p was associated with prevalent knee OA and miR-186-5p with incident knee OA and that the latter could be a potential biomarker for early OA in women. An important distinction from our study is that we examined plasma EVs and investigated all possible miRNAs sequences with NGS technology. It is worth noting that the expression levels of miRNAs in EVs are different from that in the cell cytoplasm as well as in serum and plasma[182]. Although we identified the abovementioned miRNAs in plasma EVs, no difference between patients and controls were detected.

EVs have been launched as potential biomarkers due to the distinct molecular signature that reflects the biological state of the parent cell. Together with the high stability of molecules inside the EVs and the potential to profile the EV content that can be isolated simply from body fluids, EVs and especially exosomes are considered to be a valuable source of biomarkers[183]. Over the last decade, research on EVs has grown exponentially focusing on various diseases[183,184].

Examining plasma EVs as potential biomarker for OA are relatively unexplored. The use of plasma EVs can be associated with errors. The size distribution in diameter in our study revealed two peaks, one approximately at 10 nm with a 1% intensity and a larger peak at around 200 – 300 nm with an intensity just above 7%. As exosomes usually are up to 100 nm in diameter, our findings suggest the first peak was due to the presence of exosomes and the second peak was due to the presence of the larger microvesicles and apoptotic bodies whose diameter ranging from 200 nm to above 1 μm[119].

EVs are small in size and accurate measurement can be puzzling and the protein marker of interest may be on the luminal side of the EV, making it unavailable to traditional measurement techniques. Due to the small size, EVs may express low copy numbers of the protein associated with the function or the origin under investigation. The number and composition of EVs may vary in various medical conditions.

Lately, Xu et al. have described two distinct subpopulations of exosomes[122]. Despite having identical size, morphological properties and surface markers, analysis revealed that each subpopulation had different protein profiles and miRNA-enrichment signatures, suggesting biologically different functions. These findings have been verified by Ji et. al, who identified three different subpopulations of exosomes with distinct miRNA-signatures[185]. The biological significance of these results is yet to be clarified but highlights some of the uncertainty that enclosures EVs research. If EVs are to be used as a source of biomarkers, it is of paramount importance to reach consensus in all methodological steps from sample collection to interpretation of measurements as described under the method section[118].

Our study compared all possible plasma EVs miRNA across 23 OA patients with 23 matched controls and did not detect any differences in plasma EVs miRNAs expression levels between the two groups. In fact, the close similarities in plasma EVs miRNA expression levels between persons with and without OA are remarkable and suggest that plasma EVs miRNA expression is a strictly controlled process.

Paper IV

This paper aimed to evaluate the content of miRNAs and isomiRs in plasma EVs and differences in the distribution of 5p and 3p strands in 46 persons with and without OA. We identified a total of 177 miRNAs, all with minimal differences in expression levels between the participants as described in previous section for paper III.
In regard to the distribution of 5p and 3p strands in the canonical miRNAs, both strands were detected in 32% of the canonical miRNA sequences, only 5p strands in 36% of the sequences and only 3p strands in 29% of the sequences. 3% of the sequences were without 5p and 3p strands. Overall, canonical 3p sequences were as numerous as 5p sequences.

Of the detected canonical miRNAs, 67% had isomiRs. The distribution of isomiRs showed a huge variance, ranging from 1 to 103 different isomiRs for the canonical miRNAs. The three most abundant sequences detected were the canonical forms of miR-16-5p and miR-126-3p and a miR-142-3p isomiR. Interestingly, for 52% of the canonical miRNAs, isomiRs were more abundant than the corresponding canonical form.

The 2 – 8 nt at the 5'end, denoted the seed-sequence of miRNA, are assumed to play a crucial part in binding the complementary mRNA target whereas the 3'end are believed to stabilize the miRNA[186]. Thus, any nt changes at the 5'end will give rise to a new seed-sequence with altered target, while changes at the 3'end may affect the stability of the molecule. We detected 13% changes of the isomiRs. New seeds were detected in 5.5% of the isomiRs, while 5.5% of the changes occurred at the 3'end and 2% due to substitutions within. After calculated percentage of the TMM for all identified sequences, most new seeds had very low abundance. In general, for all identified isomiRs, the two most dominant changes were addition and deletion of nt at the 3'end, albeit big variances between different miRNAs were detected. The distribution of 5p and 3p strands in isomiRs where somewhat different from that of canonical miRNAs. We found a small preponderance for only 5p strands (41%), whereas the distribution of only 3p strands and both 5p and 3p strands were equal at 27% and 26% respectively. For isomiRs, a slightly higher proportion (6%) were without 5p and 3p strands compared to miRNAs (3%).

As EVs differs from the parental cell in content such as mRNA, miRNA and isomiR signatures, it is assumed that cells do have an active mechanism of selecting cargo into EVs. To date, the selection of which miRNAs and isomiRs are incorporated into EVs are incompletely understood, although several pathways have been launched[187]. One of these possible ways for regulating miRNA incorporation into EVs is the loading of miRNA strands into the AGO protein, as AGO is found to play a role in EVs miRNA sorting[187]. Previously, it was assumed that the 5p strand was the dominant and functional strand (the guide strand) which got incorporated into the RISC, whereas the 3p strand, referred to as the passenger strand, was a minor strand with little function destined for degradation[186]. Recent studies have highlighted this and found that the 3p strand are indeed functional and that the loading of either the 5p or the 3p strand depends on the thermodynamic stability and the identity of the 5'terminal nt[188,189]. Our discovery of equal abundancy of 5p and 3p strands demonstrates that the terms "guide strand" and "passenger strands" are somewhat misleading and do not aid in

the understanding of sorting mechanism of miRNAs into EVs. Nonetheless, further studies are required to fully grasp the functional importance of this finding and the mechanisms of miRNA incorporation into EVs.

To the best of our knowledge, this is the first study that highlights the content and distribution of isomiRs in plasma EVs as well as the distribution of 5p and 3p strands across 46 humans. The discovery of the abundant presence of isomiRs in plasma EVs raises further questions about the function of these isomiRs that should be elucidated in future research.

As for paper III, our findings suggest a tight control of synthesis and secretion into EVs. As isomiRs were found so abundantly, they could be considered for future biomarker analysis and the distribution of 5p and 3p strands in miRNAs and isomiRs necessitates further analysis.

Future perspectives

- Conduct a long term RCT comparing conservative treatment, MF and ACI for the treatment of FCDs on the distal femur that includes a CEA.
- Establish a Scandinavian or nationwide cartilage registry that could monitor the prevalence, treatment errors and PROMs longitudinally.
- Conduct a study to identify to what degree health care personnel inform patients about compensation claims following treatment errors.
- Examine long non-coding RNAs in the pathogenesis and as a biomarker for early OA.
- Compare non-EVs plasma and synovial fluid miRNAs and isomiRs as a biomarker for early OA.
- Examine the distribution of isomiRs in plasma in persons with and without OA.
- Analysis of function of isomiRs.

Conclusions and implications

- MF are associated with lower costs than ACI for the surgical treatment of FCDs on the distal femur, but current CEA lack sufficient long term data to conclude which surgical treatment option is most cost-effective when treating FCDs.
- Compensation claims following surgical treatment of FCDs in Scandinavia are rare and establishing nationwide cartilage registries could increase knowledge on FCDs and enhance patient safety.
- Plasma expression levels of EVs miRNA are not different between patients with OA and without OA, and plasma EVs miRNA are not suitable as biomarkers for early OA.
- IsomiRs are found abundantly in plasma EVs and the content of isomiRs should be evaluated when analyzing plasma EVs miRNA. The equal distribution of 5p and 3p strands in miRNAs and isomiRs requires further analysis.

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Microfracture is more cost-effective than autologous chondrocyte implantation: a review of level 1 and level 2 studies with 5 year follow-up

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Abstract

Purpose Focal cartilage defects in the knee may have devastating effect on the knee joint, where two of the main surgical treatment options are microfracture and autologous chondrocyte implantation. Comparative studies have failed to establish which method yields the best clinical results. A cost-effectiveness analysis of microfracture and autologous chondrocyte implantation would contribute to the clinical decision process.

Methods A PubMed search identifying level I and level II studies with 5 year follow-up was performed. With the data from these studies, decision trees with associated service provision and costs connected to the two different techniques were designed. In addition to hospital costs, we included costs connected to physiotherapy following surgery. To paint a broader cost picture, we also included indirect costs to the society due to productivity loss caused by work absence.

Results Four high-quality studies, with a follow-up of 5 years, met the inclusion criteria. A total of 319 patients were included, 170 undergoing microfracture and 149 autologous chondrocyte implantation. The re-operation rate was 23 (13.5%) following microfracture, and 18 (12.1%) for autologous chondrocyte implantation. Both groups achieved substantially better clinical scores at 5 years compared to baseline. Microfracture was more cost-effective when comparing all clinical scores.

Conclusion Microfracture is associated with both lower costs and lower cost per point increase in patient reported outcome measures. There is a need of well-designed, high-quality randomized controlled trials before reliable conclusions regarding cost-effectiveness in the long run is possible.

Level of evidence III.

Keywords Microfracture · Autologous chondrocyte implantation · Articular cartilage lesion · Cost-effectiveness

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Abbreviations

ACI	Autologous chondrocyte implantation
AMIC	Autologous matrix-induced chondrogenesis
CCI	Characterized chondrocyte implantation
CPM	Continuous passive motion
FCD	Focal cartilage defect
HCA	Human capital approach
IKDC	International Knee Documentation Committee
KOOS	Knee injury and Osteoarthritis Outcome Score
MF	Microfracture
PROMs	Patient reported outcome measures

Introduction

The articular cartilage in joints is composed of hyaline cartilage, with optimum load bearing and friction properties. Due to limited self-repair ability, an injury to the articular cartilage will lead to permanent damage. Focal cartilage defects (FCDs) of the knee joint may lead to severe morbidity and osteoarthritis [1], and are commonly diagnosed by magnetic resonance imaging or arthroscopy. In a retrospective study of 31,516 knee arthroscopies, Curl et al. found that 63% had cartilage injuries [2]. Årøen et al. reported that 66% of 993 knee arthroscopies had cartilage lesions, with 6% having a full thickness cartilage defect [3].

There are numerous treatment options available, where all aim to reduce pain, restore function, and minimize secondary osteoarthritis. Treatments can broadly be divided into bone-morrow stimulation techniques (microfracture), osteochondral autograft or allograft transplantation and cell-based techniques (autologous chondrocyte implantation) [4, 5]. Microfracture (MF) has gained popularity over the last few decades being a minimal invasive approach with technical simplicity and low costs [6]. In addition, there are no extra laboratory expenses or secondary surgery [7]. The MF technique described by Steadman includes debridement of the defect, before an awl is used to perforate ("microfracture") the subchondral bone [8]. By this method, multipotent mesenchymal stem cells from the condyle are recruited to produce fibrocartilage filling of the defect. In contrast to this procedure, the most advanced cartilage procedure is the autologous chondrocyte implantation (ACI). This is a two-stage procedure, where the aim is to produce hyaline-like cartilage filling of the cartilage defect [9, 10]. First, small samples of normal cartilage tissue are harvested during a simple arthroscopy, and cultured in the laboratory. In the second operation, the cultured chondrocytes are re-implanted into the defect and mature into hyaline-like cartilage.

Short- and long-term studies have reported better function and less pain following knee cartilage surgery than prior to surgery [7, 11–13], but normal knee function is normally not achieved [6, 14, 15]. Based on cohort studies with at least 5 years of follow-up, no difference between the various surgical methods in regard to clinical scores, failure rates, and secondary surgeries has been found [16–19]. One study reported that MF and osteochondral autograft transplantation are equally cost-effective treatment options in a 10 year perspective [20]. Previous published studies comparing costs following MF and ACI have not analysed only high evidence studies with a minimum follow-up of 5 years, nor taken into account all the costs related to the procedure. Most notably, the costs of physiotherapy following the procedures are sparse [21, 22], whereas indirect costs to the society related to sick leave are almost absent [23].

Given the increased focus on health care efficiency and the high prevalence of FCDs on the distal femur, one should try to identify the most cost-effective treatment option to contribute to the clinical decision process for these troublesome injuries.

Previous published cost-effectiveness analysis is based on short term follow-up only. The purpose of the current study is to compare costs after 5 years between MF and ACI, based on pre-existing level 1 and level 2 studies.

Materials and methods

Miller et al. performed a cost-effectiveness analysis of cartilage injuries comparing microfracture and osteochondral autograft transplantation [20]. In the current study, we extent their method by also including costs for physiotherapy and indirect costs to society due to sick leave. A literature search was carried out in January 2017 using the database of PubMed, for clinical trials phase I and II studies comparing MF and ACI for the treatment of FCDs in the distal femur with a minimum 5 years of follow-up. Using the keywords "microfracture", "autologous chondrocyte implantation", "cartilage repair", "cartilage lesions", "mosaicplasty", "osteochondral transfer and transplantation", "osteochondral autograft" and "osteoarticular transfer system", only publications in English were included. Articles with reported evidence level I and II were included. Studies regarding the paediatric and adolescent population were excluded (as these focus on osteochondritis dissecans). As long as they met inclusion criteria, studies comparing other cartilage procedures were included.

According to the standard methods for economic evaluation of health care programs, decision trees following MF and ACI as previously described by Drummond et al. were constructed [24]. Terminal endpoints were either success or failure, where the latter was defined as pain or loss of function which required revision surgery. Based on the decision tree and clinical experience regarding service provision in the two different alternatives, treatment paths were constructed for MF and ACI, respectively. The cost data were taken from a local orthopedic hospital in Norway, and

verified via personal communication with other orthopedic hospitals in the country. Direct costs including physiotherapy was first calculated, and second indirect costs related to sick leave was included. Hospital costs (unit prices) were based on a cost-per-patient calculation model, which is an established standard for calculating patient-level costs in hospitals [25] (Table 1).

The costs of revision surgery were calculated for the specific procedures (diagnostic arthroscopy, MF, mosaicplasty, ACI, high tibial osteotomy, and total knee arthroplasty), and included costs of a magnetic resonance imaging and a return visit. For the costs of one overnight stay in an orthopedic ward, a Norwegian estimate is €620 [26]. The length of hospital stays following MF and ACI varies, where stays up to 4 days have been reported [23, 27]. In this study, lengths of stay are set to 1 day for MF and 3 days for ACI which corresponds to both clinical practice and the current literature [28] (Table 2). In regard to revision surgery, we assumed hospital stays of 1 day following diagnostic arthroscopy and mosaicplasty, and 3 days for high tibial osteotomy and total knee arthroplasty.

In regard to postoperative physiotherapy, there is no consensus regarding frequency or duration. Our assumptions are based on clinical experience and personal communications. After ACI, Brittberg recommends physiotherapy twice weekly for 24 weeks (personal communication), while Robert LaPrade at Steadman's clinic recommends physiotherapy twice weekly for 12 weeks following MF (personal communication). When estimating the costs of physiotherapy following revision surgery, we assumed physiotherapy twice weekly for 12 weeks after diagnostic arthroscopy, MF and mosaicplasty, and twice weekly for 24 weeks for ACI, high tibial osteotomy and total knee arthroplasty. The unit cost of one session physiotherapy is €30 (The Norwegian Physiotherapist Association). No brace was included in the costs.

A human capital approach (HCA) was employed to calculate indirect costs (productivity loss). In HCA, the loss to the society is estimated from the income normally earned by the patients [29]. The idea is that the employees' wages provide an estimate of the value their labour contributes to the economy, and labour that is lost due to sick leave is assumed to reduce the society's total productivity accordingly. A total of 5 days off work is expected following MF surgery, and 15 days following ACI [23]. Based on data from Statistics Norway (2016), a fulltime employee (both genders) aged 30–34 years earns €4667 per month, or €215 for each day absent from work [30]. This age range

Table 1 Total cost primary surgery per patient

Variable	Unit price (€)	Cost (units)	
		MF	ACI
Initial consult	95	95 (1)	95 (1)
Surgery and material		1749	3498
Cell culture	4050		4050
Outpatient follow-up visit	35	70 (2)	70 (2)
Hospital stay (each night)	620	620 (1)	1860 (3)
Physiotherapy	30	720 (24)	1440 (48)
Direct costs		3254	11,013
Indirect costs (absent from work)	215	1075 (5)	3225 (15)
Total costs		4329	14,238

All costs in Euros (€) per patient

MF microfracture, *ACI* autologous chondrocyte implantation, *N/A* not applicable

Table 2 Total cost revision surgery per patient

Variable	Unit price(€)	Cost (units)					
		DA	MF	MOS	ACI	HTO	TKA
Cost revision surgery							
Return visit	95	95 (1)	95 (1)	95 (1)	95 (1)	95 (1)	95 (1)
MRI	198	198 (1)	198 (1)	198 (1)	198 (1)	198 (1)	198 (1)
Revision surgery and material		1749	1749	3098	3498	8030	10,563
Cell culture	4050				4050		
Outpatient follow up	35	70 (2)	70 (2)	70 (2)	70 (2)	70 (2)	70 (2)
Hospital stay	620	620 (1)	620 (1)	620 (1)	1860 (3)	1860 (3)	1860 (3)
Physiotherapy	30	720 (24)	720 (24)	720 (24)	1440 (48)	1440 (48)	1440 (48)
Direct cost		3452	3452	4801	11,211	11,693	14,226
Indirect cost (absent from work)	215	1075 (5)	1075 (5)	1075 (5)	3225 (15)	3225 (15)	3225 (15)
Total cost revision surgery		4527	4527	5876	14,436	14,918	17,451

All costs in Euros (€) per patient

MRI magnetic resonance imaging, *DA* diagnostic arthroscopy, *MF* microfracture, *ACI* autologous chondrocyte implantation, *MOS* mosaicplasty, *HTO* high tibial osteotomy, *TKA* total knee arthroplasty

corresponds to the average age of the patient population. Assessing the indirect costs (sick leave) following revision surgery, we assumed 5 day off work for diagnostic arthroscopy, MF and mosaicplasty, and 15 day off work for ACI, high tibial osteotomy and total knee arthroplasty.

Total costs at 5 years are calculated by summing primary costs and costs for revision surgery.

By comparing total costs and the weighted average of the reported outcome measures, we calculated the costs related to a 1-point increase in each of the reported PROM values following MF and ACI. All costs were converted to 2017 Euros based on the Norwegian consumer price index.

A sensitivity analysis was performed to calculate alternative values to see how sensitive the end result is for the choice of value on different variables. Guidelines often discount costs at a 3% annual rate, considered to start at 5 years [31]. Since our study has a follow-up of 5 years, the costs were not discounted.

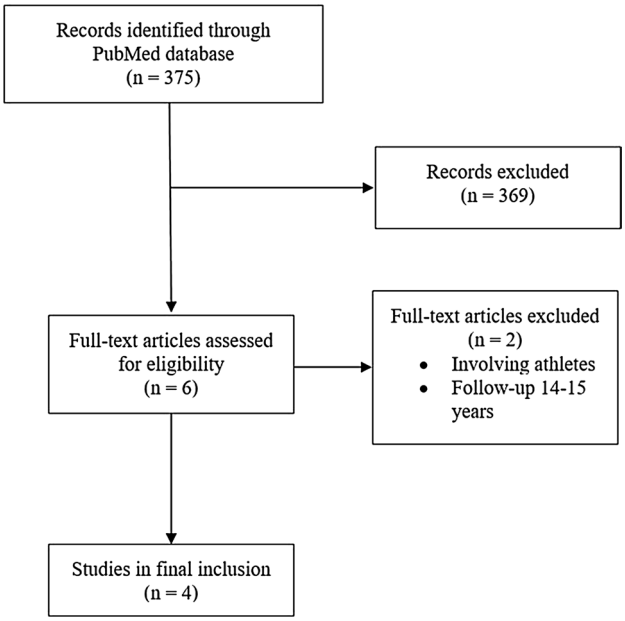

Fig. 1 Flow diagram of article selection included in the study

Results

Six studies were identified [16–19, 32, 33] (Fig. 1), corresponding to three systematic reviews [11, 34, 35]. Three studies compared MF with ACI using periosteum [16, 18, 32], two compared MF with scaffold ACI [17, 33], whereas one compared MF with characterized chondrocyte implantation (CCI) [19]. One study involving high level athletes did not report failures, and was excluded [33]. One author had published results both after 5 and 14–15 years [16, 32], but only the 5 year results were included. Hence, 4 articles with 319 patients (208 males, 65%) formed the basis for comparison of clinical scores schemes, failure rates and revision surgeries [17–19, 32] (Table 3).

170 patients underwent MF, and 149 ACI. Patients in the two groups were 32.1 (MF) and 33.1 (ACI) years, with lesion sizes 2.5 cm^2 (MF) and 3.2 cm^2 (ACI). Based on the decision trees, 147 (86.5%) in the MF group, and 131 (87.9%) in the ACI group achieved success at 5 years (Figs. 2, 3).

One study did not specify treatment failure [32]. One study reported re-intervention rates, but did not specify the revision procedure [19]. For our cost analysis, we assumed the non-specific revisions as diagnostic arthroscopies (ten in the MF group and seven in the ACI group).

MF had direct costs of €3254 at baseline (Table 1), rising to €3892 at 5 years (Table 4), while ACI was €11,013 at baseline, increasing to €11,558 at 5 years. When we included productivity loss due to sick leave, MF had total initial costs of €4329 rising to €5150 at 5 years. For ACI, the total costs at baseline and at 5 years were €14,238 and €14,941, respectively. Total costs connected to revision surgery were slightly higher in the MF group (€821) compared to ACI (€703) (Table 4).

Different validated patient reported outcome measures (PROMs) were used. The Tegner score was used in three studies [17, 18, 32], the Lysholm score was reported in two [18, 32], whereas the visual analogue scale (VAS) [32], Short Form 36 (SF-36) [32], Hospital for Special Surgery (HSS) [18], the Knee Injury and Osteoarthritis Outcome Score (KOOS) [19] and the International Knee Documentation Committee (IKDC) [17] were reported in one study each. Comparing the weighted average of the preoperative

Table 3 Summary of the included articles

Level	References	Technique	Patients	PROMs
I	Knutsen et al. [32]	MF—P-ACI	40–40	VAS, Lysholm, Tegner, SF-36
II	Lim et al. [18]	MF—P-ACI	29–18	Lyshom, Tegner, HSS Knee score
II	Kon et al. [17]	MF—scaffold ACI	40–40	IKDC, Tegner
I	Vanlauwe et al. [19]	MF—CCI	61–51	KOOS

MF microfracture, *P-ACI* autologous chondrocyte implantation using periosteum, *PROMs* patient reported outcome measures, *VAS* visual analogoue scale, *SF-36* short form 36, *HSS* hospital for special surgery, *KOOS* Knee Injury and Osteoarthritis Outcome Score, *IKDC* International Knee Documentation Committee, *Ref* reference number

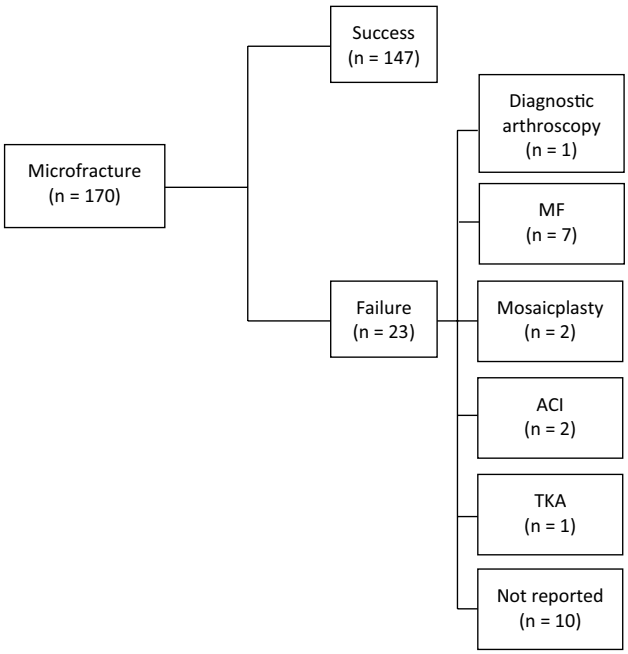

Fig. 2 Microfracture decision tree. *n* number of patients, *MF* microfracture, *ACI* autologous chondrocyte implantation, *TKA* total knee arthroplasty

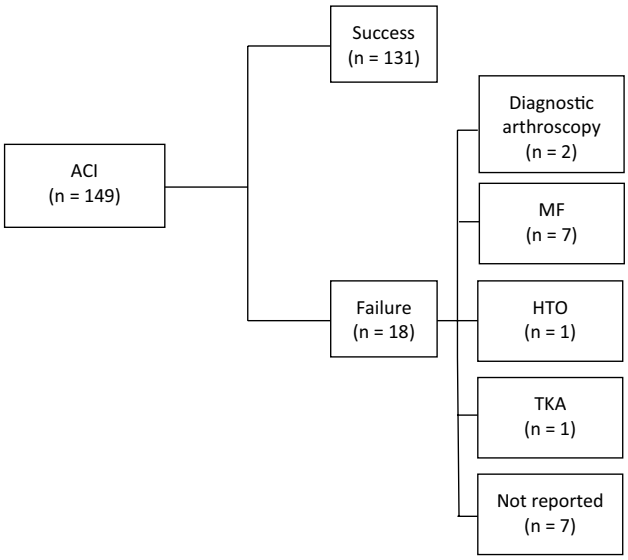

Fig. 3 Autologous chondrocyte implantation decision tree. *n* number of patients, *MF* microfracture, *HTO* high tibial osteotomy, *TKA* total knee arthroplasty

PROMs with the weighted average of the PROMs after 5 years, all reported statistically clinical improvement for both MF and ACI [34, 36]. Based on the weighted average of the PROMs, a comparative cost-effectiveness analysis was carried out given a 1-point increase on each of the reported clinical scores for total costs at 5 years. For all measures, a 1-point increase in clinical scores had lower costs for MF than for ACI at 5 years (Table 5).

The sensitivity analysis showed that a 66% reduction in the total costs following ACI or a 190% increase in the total costs of MF led to equivalent total costs at 5 years. Comparing only primary direct costs, a reduction in costs of 70% after ACI, or a 239% increase in costs after MF would lead to equivalent costs at baseline. Assuming identical costs for hospital stay, physiotherapy and sick leave after the primary surgery, an increase in costs of 69% following MF and a decrease in costs of 41% after ACI would lead to identical total costs after the primary surgery.

Discussion

The most important finding of the present study was that MF is more cost-effective than ACI for the treatment of FCDs in the distal femur with 5 year follow-up. The main difference in total costs is related to the primary surgery, where MF is less expensive than ACI. Costs following revision surgery are however lower in the ACI group, respectively, €703 (ACI) and €821 (MF) per patient.

The included studies have demonstrated that the clinical scores are statistically significantly better at 5 years compared to pre-surgery for both methods. Not all studies reported variances or standard deviations, so we were unable to calculate a precise *p* value that demonstrates that the difference in cost-effectiveness is statistically significant. However, given the large differences in costs per point improvement between MF and ACI, it is unlikely that our findings are purely coincidental.

In a recent study comparing MF, osteochondral autograft transplantation and ACI, Schrock et al. reported that MF was the most cost-effective treatment option for chondral lesions in the knee, confirming our findings [37]. In contrast, Mistry et al. reported ACI to be cost-effective compared to MF [22]. When calculating costs, Mistry et al. assumed ACI to be performed as outpatient surgery with a total of six outpatient follow-ups, while MF was assumed to be inpatient surgery. Because ACI is a far more invasive procedure than MF, we assumed 3 days of hospitalization and two outpatient follow-ups after ACI, and 1 day of hospitalization following MF. This is the most important reason why our results differ to Mistry et al. In addition, our study adds costs related to sick leave.

There is a wide variation between surgeons in relation with indication for surgery, preferred surgical technique, postoperative physiotherapy, and outcome assessment [38]. Some have suggested that MF should be performed as first procedure in FCDs on the femur due to the simplicity of the procedure and the associated low costs [6, 7, 39]. MF has,

Table 4 Total costs at 5 years

		MF (patients)	ACI (patients)
Direct costs			
Primary surgery		553,180 (170)	1,640,937 (149)
Revision surgery			
	Unit price (€)		
DA	3452	3452 (1)	6904 (2)
MF	3452	24,164 (7)	24,164 (7)
MOS	4801	9602 (2)	N/A
ACI	11,211	22,422 (2)	N/A
HTO	11,693	N/A	11,693 (1)
TKA	14,226	14,226 (1)	14,226 (1)
Not reported	3452	34,520 (10)	24,164 (7)
Direct costs		661,566 (170)	1,722,088 (149)
Direct costs per patient		3892	11,588
Total costs			
Primary surgery		735,930 (170)	2,121,462 (149)
Revision surgery			
	Unit price (€)		
DA	4527	4,527 (1)	9054 (2)
MF	4527	31,689 (7)	31,689 (7)
MOS	5876	11,752 (2)	N/A
ACI	14,436	28,872 (2)	N/A
HTO	14,918	N/A	14,918 (1)
TKA	17,451	17,451 (1)	17,451 (1)
Not reported	4527	45,270 (10)	31,689 (7)
Total costs revision surgery		139,561 (170)	104,801 (149)
Revision surgery costs per patient		821 (23)	703 (18)
Total costs		875,491 (170)	2,226,263 (149)
Total costs per patient		5150	14,941

The non-specific revisions listed as not reported were assumed as diagnostic arthroscopy in the cost analysis. The cost calculations after primary surgery is based on Table 1

All costs in Euros (€)

DA diagnostic arthroscopy, *MF* microfracture, *MOS* mosaicplasty, *ACI* autologous chondrocyte implantation, *HTO* high tibial osteotomy, *TKA* total knee arthroplasty, *N/A* not applicable

therefore, become the gold standard to which other methods have been compared in clinical trials [27, 40].

The effect of the cartilage lesion size on symptoms is poorly investigated. Some authors recommend ACI for cartilage lesions larger than 4 cm^2 [7, 41], but the literature is unclear on lesions ranging from 2 to 4 cm^2 [42]. The average lesion sizes in our study were slightly different between the groups (2.5 cm^2 for MF and 3.2 cm^2 for ACI), which probably do not affect our result. Recent research on anterior cruciate ligament reconstruction in combination with articular cartilage injury has found the effect of the lesion size to be minor [43].

Kon et al. compared MF with scaffold ACI and found a small clinical benefit in favour of ACI after 5 years [17]. Knutsen et al. reported no clinical significant differences comparing MF and ACI after 14–15 years [16]. Radiological early signs of osteoarthritis were found in 48% in the MF group and 57% in the ACI group. In a long-term perspective, this could affect the cost-effectiveness of these two methods.

There are technological advances both within MF and ACI. Nanofracture, scaffolds, and autologous matrix-induced chondrogenesis are gaining popularity, and may give a different clinical and cost picture of microfracture derived procedures. Scaffold may induce significantly higher costs when comparing MF with other cartilage procedures. Published papers on ACI are mainly based on first generation procedures, but second and third generation ACI have now been implemented both in clinical trials and practice. However, long-term results are not yet available [44]. Besides, the use of characterized chondrocytes implantation may yield different results than ACI. A third factor is that ACI is performed as a two-stage procedure. The development of

Table 5 Cost per 1-point improvement in the patient reported outcome measures for total cost at 5 years

PROM	PROM difference Baseline—5 years	Costs per point Improvement per patient (€)
VAS		
MF	29	178
ACI	28	534
Lysholm		
MF	26	198
ACI	19	786
Tegner		
MF	1.8	2861
ACI	2.8	5336
SF-36		
MF	10	515
ACI	7	2134
HSS		
MF	9.4	548
ACI	10.0	1494
KOOS		
MF	14.1	365
ACI	21.2	705
IKDC		
MF	30	172
ACI	42	356

All costs in Euros (€)

PROM patient reported outcome measure, *MF* microfracture, *ACI* autologous chondrocyte implantation, *VAS* visual analogue scale, *SF-36* short form 36, *HSS* hospital for special surgery, *KOOS* Knee Injury and Osteoarthritis Outcome Score, *IKDC* International Knee Documentation Committee

one-stage procedures may yield different health economic effects than traditional ACI, and would probably lower the costs substantially [23].

This study has limitations. Studies with evidence levels 1 and 2 comparing MF and ACI with a minimum follow-up at 5 years are few, leading to relatively small study populations. This may lead to bias and affect the results published in this article. Yet, to this date, these are the only high-quality studies with 5 year follow-up.

Another limitation is the fact that the MF group had slightly smaller lesions and, therefore, might represent patients which are more responsive to physiotherapy after surgery. Supervised physiotherapy has also been shown to be effective together with debridement of the lesion [45], and our study cannot determine which method yield better clinical results.

Knutsen et al. published the SF-36 and Tegner score only for the success patients, and not for the failures [32]. This may lead to an overestimation of these scores, as we must assume that failures would have lower scores than the successes.

Physiotherapy before surgery and costs related to independent training are not included in our calculations because we assume them to be similar for the two groups. A wide range of postoperative physiotherapy protocols after MF and ACI exists. Some permit weight-bearing, while others use continuous passive motion (CPM) [13, 45–48]. These differences may affect the cost calculations.

Ten patients in the MF group and seven in the ACI group were re-operated, and the procedure was assumed to be diagnostic arthroscopies. If we assumed another re-operation procedure, this would affect the cost estimates. When estimating the costs related to hospital stay, work absence, and physiotherapy after revision surgery, we used the cost estimates from primary surgery, which may be an underestimation.

In regard to capital costs, account investments and orthopedic skills were not taken into account, i.e., we have assumed that hospitals can switch between MF and ACI, which in practice is not the case.

The unit prices employed in our calculations is extracted from a local orthopedic hospital, and confirmed with other orthopedic hospitals in Norway, which may limit the transferability of the study. An international cost analysis is difficult to perform because different countries face different institutional and financial constraints, including different unit prices. On the other hand, their assumption regarding service provision related to surgery, postoperative physiotherapy and sick leave are comparable to other studies, thereby giving a certain degree of transferability globally [22, 23, 37].

The results are based on 5 year follow-up. In light of cartilage pathologies, this may be sparse. However, failures usually occur within 2–3 years after the initial surgery [49, 50], so our timeline seems sufficient to capture failures. Furthermore, none of the included studies compared surgical treatment with conservatively treatment, so the true effect of surgery is in fact not known [35, 51]. High-quality studies with follow-up exceeding 5–10 years with a conservative control group are needed to be able to draw conclusions on this painful and morbid disease. Treatment of FCDs is expensive for the society, and our study may contribute to the decision process in clinical practice. This study has a broader perspective than previous cost analyses and should be of particular interest for orthopedic surgeons of this particular knee injury.

Conclusion

There is evidence for the benefits of cartilage repair surgery using MF and ACI based on the 5 year results published

when evaluating health costs related to the procedures. The MF procedure is more cost-effective than ACI based on published 5 year results, but there is a need of well-designed, high-quality randomized controlled trials with long-term results before safe conclusions can be made.

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Author contributions TFA performed literature search, drafted and edited the article. PHR co-drafted and co-edited the article. HL co-drafted and co-edited the health economic method, results and discussion. OBL gave critical review of the manuscript. AA launched the hypothesis of the study with study design and gave critical review of the manuscript and provided funding. All authors made contributions to design, was involved in the drafting and read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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Ethical approval The manuscript uses clinical data based on previous published literature, all approved by an ethics committee. This study is in accordance with the ethical standards of the 1964 Declaration of Helsinki.

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Compensation claims after knee cartilage surgery is rare. A registry-based study from Scandinavia from 2010 to 2015

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Abstract

Background: Focal cartilage defects (FCDs) in the knee joint has a high prevalence. A broad range of treatment options exists for symptomatic patients. Knowledge of patient compensation claims following surgical treatment of FCDs is missing. The purpose of this study is to evaluate compensation claims filed to the Scandinavian registries for patient compensation following treatment of FCDs in the knee joint from 2010 to 2015 and identify possible areas of improvement.

Methods: A cross-sectional study design was used to obtain all complaints following surgical treatment of FCDs from the Scandinavian registries from 2010 to 2015. Data such as age, gender, type of treatment, type of complaint, reason of verdict and amount of compensation were collected and systematically analyzed.

Results: 103 patients filed a compensation claim. 43 had received debridement (41.7%), 54 microfracture (MF) (52.4%), 3 mosaicplasty (2.9%) and 3 autologous chondrocyte implantation (ACI) (2.9%). Of the 103 claims, 36 were granted (35%). 21 following debridement (58.3%), 13 after MF (36.1%), 1 following mosaicplasty (2.8%) and 1 after ACI (2.8%). The most common reason for complaint was infection (22.1%), of which 89% were granted. The average compensation was €24.457 (range €209 – €458.943).

Conclusion: Compensation claims following surgical treatment of knee cartilage injuries in Scandinavia are rare. Establishing nationwide cartilage registries can add further knowledge on this troublesome disease.

Keywords: Articular cartilage, Microfracture, Autologous chondrocyte implantation, Compensation claim

Background

Focal cartilage defects (FCDs) in the knee joint is a high prevalence injury that may cause pain and reduced function, with the risk of early onset osteoarthritis [1–3]. Various surgical treatment options are available. The goal of operative treatment is to restore the articular cartilage and reduce symptoms and minimizing the risk of osteoarthritis [4, 5]. Surgical treatment relieves symptoms, but regardless of surgical procedure, the majority of patients do not achieve normal knee function [6–8]. No method or treatment has proved to be superior to any other, and there is currently no gold standard or consensus on what constitutes the best treatment for FCDs of the knee [9–11].

Orthopedic surgery is one of the medical specialties with the highest rate of compensation claims following medical treatment [12]. Consequently, there is an increased interest in compensation claims related to orthopedic surgery [13, 14]. Previous studies have mainly

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reported compensation claims following hip and knee arthroplasty and spine disorders [15, 16]. One study has reported malpractice litigation following arthroscopic surgery in general, but to the best of our knowledge, no study has previously reported compensation claims following FCDs in the knee specifically [17].

The purpose of this study is to evaluate compensation claims filed to the Scandinavian registries following surgical treatment of FCDs in the knee joint from 2010 to 2015 and identify possible areas of improvement. We hypothesized that compensation claims are more frequent after debridement and microfracture (MF) compared to mosaicplasty and autologous chondrocyte implantation (ACI).

Methods

Data source

In Scandinavia, compensation claims for injuries in connection with medical treatment are handled by nationwide systems. The compensation principle in these nations is a no-blame system based on the principle of avoidability (i.e. if the injury sustained during treatment was avoidable). A no-blame compensation principle separates the compensation issue from legal malpractice, permitting most indemnity cases in Scandinavia to be settled outside the judicial system. In Norway, the complaints are handled by the Norwegian System of Patient Injury Compensation (NPE) [18]. Patients can appeal against a decision to the Patient Injury Compensation Board, which is under the Ministry of Health. In Sweden, the claims are processed by the National Swedish Patient Insurance Company, a mutual company owned by the counties [19]. In Denmark, the Patient Insurance Association handles claims concerning malpractice and injuries, as well as injuries caused by medical products [20].

In all three systems, compensation is only considered if three conditions are met [18]. Firstly, the injury must have been caused by the examination, diagnosis, treatment (or lack of treatment) or follow-up of the condition. The treatment (or lack thereof) must be deemed erroneous or substandard compared to current treatment guidelines. If the reason for complaint is considered to be a consequence of the primary injury, and not the treatment, compensation is not granted. There is one exception to this rule (the reasonability rule). This exception permits compensation to be granted after rare and serious complications even if no treatment failure can be identified. Secondly, the patient must have led a substantial financial loss in excess of what would otherwise be expected. Thirdly, the claim must be put forward within a reasonable time (currently set to 10 years in Sweden and three years in Denmark and Norway). These similar conditions enable us to combine data from all three Scandinavian countries in our analysis.

Participants

Data from the Danish, Norwegian and Swedish nationwide registries were obtained from each of the respective national registries. Patients of both genders and of any age who filed a compensation claim following articular cartilage surgery of the knee from 2010 to 2015 were considered for inclusion. The nationwide databases were searched for a predefined set of diagnosis and surgical procedures using the International Statistical Classification of Diseases and Related Health Problems 10th Revision (ICD-10) and the NOMESCO Classification of Surgical Procedures (NCSP) Version 1.14 [21, 22] (Table 1). The potential patient files were subsequently screened by the corresponding author, identifying patients who had been treated for an isolated cartilage defects of the knee. The surgical notes were then reviewed before final inclusion (Fig. 1).

The age, gender and nationality of the patients were collected, together with the type of treatment, type of complaint and the amount of compensation in granted cases. The reasons given for granted or rejected claims were reviewed and systematically analyzed.

Statistics

Mean, median and standard deviation were calculated for continuous variables. Categorical data were presented in frequencies and cumulative frequencies. Groups were compared using the independent t-test or the Chi-square test. A p-value < 0.05 was considered statistically significant. The analysis was performed using IBM SPSS Statistics v25.

Results

We identified 103 compensation claims put forward to the registries following articular cartilage surgery in the knee from 2010 to 2015 (Fig. 1). There was a slight decrease in claims for compensation the last two years of the study period (Fig. 2). Most claims were put forward to the Danish registry (Fig. 3).

The average age at the time of surgery was 38.6 years (11–71). 62 (60.2%) claims were put forward by females (Table 2). Claims following debridement (43, 41.7%) and MF (54, 52.4%) was far more common than following mosaicplasty (3, 2.9%) and ACI (3, 2.9%).

Of the 103 claims, 36 were granted (35%). There was no statistically significant difference in granted claims between males and females (15/41 versus 21/62, $p = 0.8$). 21 of the patients with granted claims were treated with debridement (58.3%), 13 with MF (36.1%), 1 with mosaicplasty (2.8%) and 1 underwent ACI (2.8%). Infection (22.2%), pain (16.7%), delayed diagnosis or

Table 1 Overview of the predefined diagnosis and surgical procedures using the ICD-10 and NCSP codes

Diagnosis	Surgical procedures
M17 Gonarthrosis	NGA11 Endoscopic exploration
M22.4 Chondromalacia patella	NGA12 Open exploration
M23.4 Loose body in the knee	NGF21 Endoscopic fixation of corpus librum
M23.8 Other internal derangements of knee	NGF22 Open fixation of corpus librum
M23.9 Internal derangement of knee, unspecified	NGF31 Endoscopic resection of corpus librum
M24.1 Other articular cartilage disorder	NGF32 Open resection of corpus librum
M24.8 Other specific joint derangements, not elsewhere classified	NGF91 Other endoscopic procedure on synovia or articular cartilage
M24.9 Joint derangements, unspecified	NGF92 Other open procedure on synovia or articular cartilage
M25.5 Pain in joint	NGG29 Other arthroplasty without prosthesis
M25.8 Other specified joint disorders	NGG99 Other excision, reconstruction or arthrodesis of knee
M25.9 Joint disorder, unspecified	NGH41 Endoscopic removal of corpus librum
M92.4 Juvenile osteochondrosis, unspecified	NGH42 Open removal of corpus librum
M92.8 Other specified juvenile osteochondrosis	NGH91 Other endoscopic procedure
M92.9 Juvenile osteochondrosis, unspecified	NGH92 Other open procedure
M93.2 Osteochondritis dissecans	NGK09 Excision of bony fragment in knee
M93.8 Other specified osteochondropaties	NGK19 Resection or excision of bone in knee
M93.9 Osteochondropathy, unspecified	NGK29 Fenestration or drilling of bone in knee
S83.3 Tear of articular cartilage of knee, current	NGN09 Autotransplantation of bone to knee
	NGN49 Transplantation of cartilage, periost or fascia to knee
	NGN99 Other transplantation to knee
	YNA20 Removal of cartilage for transplantation
	ZZG00 Cartilage transplantation

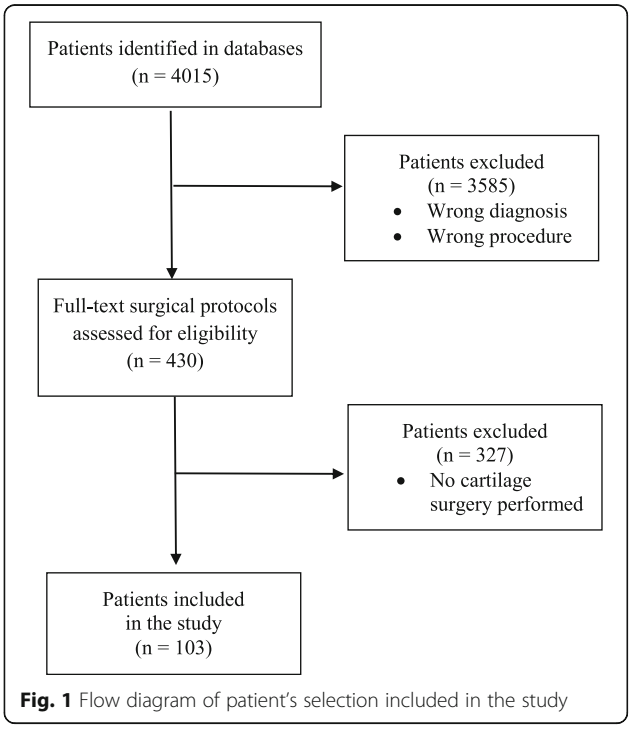

Fig. 1 Flow diagram of patient's selection included in the study

treatment (13.9%), treatment failure (11.1%) and numbness (11.1%) dominated patients' reasons for complaints (Table 3).

Of the patients claiming for compensation due to infection, 89% were granted, whereas for pain, only 14% of the claims were granted.

29 patients received compensation related to surgery (such as infection or inadequate surgical technique), whereas 7 patients received compensation unrelated to surgery (such as delayed diagnosis or treatment or failure of medical equipment (Table 4).

All 8 patients given compensation due to surgical site infection underwent debridement. One patient who underwent debridement was granted compensation due to an infected peripheral vein catheter.

The majority of claims were rejected because good clinical practice was followed or because no causal connection was found. Three claims were rejected because there was no financial loss.

Complaints from public hospitals were compensated more often (31/89) than complaints from private hospitals (5/14) ($p = 0.004$).

A total of €807.086 has been paid in compensation with an average payment of €24.457. In 3 cases the amount of compensation had not yet been settled. The

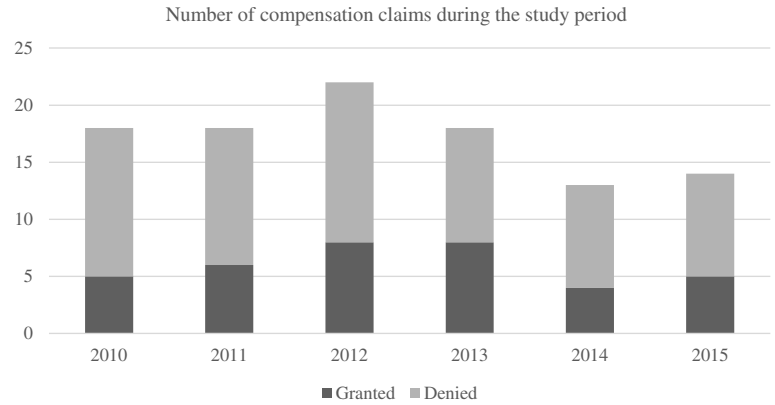

Fig. 2 Complaints filed to the Scandinavian registries following surgical treatment of focal cartilage defects in the knee joint between 2010 and 2015

median compensation was €5652, with range €209 - €458.943. The skewed distribution of compensation was caused by one patient, who was granted compensation 10 times higher than the second highest compensation. This patient was a 47-year-old female who sustained a hospital-acquired infection following debridement. This led to almost 2.5 years of sick-leave, explaining the high compensation.

Discussion

This study highlights the epidemiology of patient compensation claims following articular cartilage surgery in the knee joint over a six years period. The main reasons for compensations were inadequate surgical technique (no further explanation was accessible), hospital-acquired infection, nerve injury and delayed diagnosis or treatment. Most claims filed for compensation due to hospital-acquired infection was granted compensation, all following arthroscopic debridement. Pain was a common reason for patients' complaint, but is usually not a valid cause of compensation by itself. Our study also finds that women more often file a claim than men [23]. There was no mortality recorded or claims due to wrong-sided surgery.

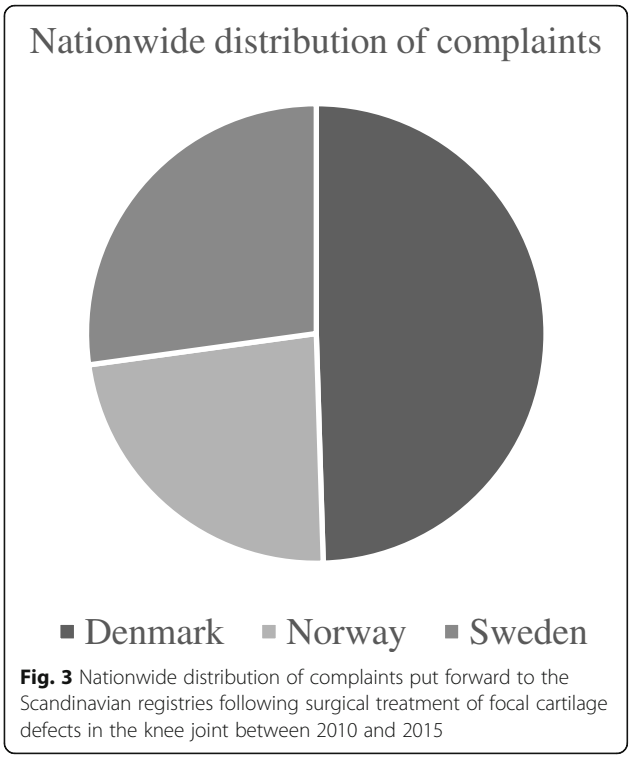

Fig. 3 Nationwide distribution of complaints put forward to the Scandinavian registries following surgical treatment of focal cartilage defects in the knee joint between 2010 and 2015

There was a surprisingly low number of compensation claims identified in Scandinavia in the study period. The true incidence of cartilage procedures is unknown, but the incidence seems to be increasing [24]. Merkely et al. stated that more than 200,000 cartilage procedures were performed annually in America [25], and Engen reported approximately 2500 cartilage procedures are performed annually in Norway [26]. This yields approximately 45, 000 cartilage procedures in Scandinavia during the study period. Based on these numbers, one should expect a higher number of compensation claims. We identified 103 compensation claims over a six-year period, an average of 17 complaints annually. This is substantially lower than the findings of Randsborg et al. who identified 24 compensation claims yearly following anterior cruciate ligament reconstruction in Norway alone [27].

We found more compensation claims in Denmark, despite the fact that Sweden has about twice the population size. The reason for this is unclear. We believe it reflects cultural differences, rather than a real difference in the quality of cartilage surgery between the respective countries. In fact, it might indicate that Denmark has a better system of detecting patient injury claims.

Since the introduction of ACI two decades ago [28], this procedure has gained popularity both routinely and in clinical trials, as is the case for mosaicplasty [6, 11,

Table 2 Age and gender partitioned by declined or rejected claims following surgical treatment of focal cartilage defects in the knee joint

	Declined, ***n*** = 67 (65%)	Granted ***n*** = 36 (35%)	
Age, mean (SD, range)	38.5 (10.7, 11–71)	38.8 (12.1, 13–55)	0.93
Females, n (%)	41 (61.1%)	21 (33.9%)	0.77

29]. Nevertheless, compensation claims following mosaicplasty and ACI are almost absent in our material covering three countries for six years. Only two cases of compensation following mosaicplasty or ACI were found. These findings are in line with previous studies stating that major complications following mosaicplasty and ACI are very rare [30–33]. Debridement and MF are low-cost and relatively simple procedures available in smaller hospitals and private clinics that cannot offer the more advanced cartilage procedures, such as mosaicplasty and ACI, which requires highly specialized institutions. The total numbers of debridement and MF performed annually is much higher than mosaicplasty and ACI [26]. This explains the large predominance of complaints by debridement and MF.

Lack of communication and poor patient expectation management are well-known risk factors for compensation claims [34]. It is possible that patients scheduled for mosaicplasty or ACI in highly specialized knee units are better prepared and well informed prior to surgery, and might receive better follow-up, than patients operated in smaller clinics. Furthermore, mosaicplasty and ACI are often considered salvage procedures when simpler interventions have failed. This might alter the patient expectations to these more complex knee surgeries, which again affects the threshold for filing a compensation claim.

Table 3 Patients' reasons for complaint in 36 granted claims following surgical treatment of focal cartilage defects in the knee joint

Reason for complaints (granted)	***N*** = 36 (%)
Infection	8 (22.2%)
Pain	6 (16.7%)
Delayed diagnosis or treatment	5 (13.9%)
Treatment failure	4 (11.1%)
Numbness	4 (11.1%)
Spinal headache	2 (5.6%)
Stiffness	1 (2.8%)
Swelling	1 (2.8%)
Lack of information	1 (2.8%)
Infected peripheral vein catheter	1 (2.8%)
Failure of medical equipment	1 (2.8%)
Deep vein thrombosis	1 (2.8%)
Frozen shoulder	1 (2.8%)

Although most complications were related to the surgery, 2 were caused by the anesthesia. This is a reminder that surgery also included risks unrelated to the procedure itself.

Ohrn et al. showed that 23% of all compensation claims to the National Swedish Patient Insurance Company were attributed to orthopedic surgery, whereas Bjerkreim reported that 47% of all compensation claims filed to the NPE were after orthopedic treatment [35, 36]. National health oversights in Scandinavia have reported that patients' complaints have increased in all three countries in recent years [37]. From 2005, there has been approximately a 10% annual increase in compensation claims.

Although patients have become more aware of the possibility of applying for compensation, our findings indicate that complaints following knee cartilage surgery are fewer than anticipated. The reason for this may be diverse. Perhaps the surgically treated cartilage patients are so troubled by their knee that they have low expectations. Or, although unlikely, the surgery is successful for most of the patients. Another possible reason is the lack of information from health care professionals regarding the opportunity to apply for compensation.

The amount of compensation following arthroscopic surgery varies greatly between countries. In their study of medical malpractice litigation following knee arthroscopy, Shah et al. found an average settlement of $848.331 (€733.486) [17]. We found an average compensation of €24.457. This is almost exactly the same amount of compensation granted following anterior cruciate ligament reconstruction in Norway (€24.200) [27]. This indicates that compensation amount is substantially lower in Scandinavia than in the United States.

Table 4 Registries' reasons for compensation in 36 granted claims following surgical treatment of focal cartilage defects in the knee joint

Reason for granted compensation	N = 36 (%)
Inadequate surgical technique	12 (33.3%)
Hospital-acquired infection	9 (25.0%)
Nerve injury	5 (13.9%)
Delayed diagnosis or treatment	4 (11.1%)
Treatment failure	3 (8.3%)
Spinal headache	2 (5.6%)
Failure of medical equipment	1 (2.8%)

The study from the United States by Shah and colleagues reported medical malpractice litigation following arthroscopic surgery [17]. Over 29 years, they reported 162 litigations following knee arthroscopy, yielding less than six litigations annually. This is substantially lower than our findings of 17 compensation claims annually, and they did not specify which treatment was given. Shah. et al. found that 64% of the claims were rejected, similar to our findings. They reported musculoskeletal complaint (listed as chronic pain, stiffness and unsatisfactory result), infection and deep vein thrombosis as the three main reasons for compensation claims. Different from our finding, Shah reported 19 deaths and 10 cases of wrong-sided surgery, whereas we registered no deaths or wrong-sided surgery. Our study differs from theirs as we only report compensation claims following treatment of FCDs and have excluded other common arthroscopic procedures such as ligament reconstruction and meniscal procedures. On this basis, our findings supplement the results of Shah et al. and add further knowledge in compensation claims following arthroscopic surgery and FCDs in particular.

The Scandinavian countries use the no-blame principle for practitioners in handling compensation claims, eliminating the fault criterion. This implies that no data is shared with the regulatory authorities, and cases are usually handled outside the legal system where the insurance provider recovers the cost of a claim from the liable party. The no-fault approach system is not unique in Scandinavia, as this is found in Finland, France, New Zealand and two American jurisdictions (Florida and Virginia) [38, 39]. The opposite of a non-fault claim is the court-based tort law system, where the liable party is responsible for the cost of a claim based on the fault criterion. This system is among other countries used in the United Kingdom and most American jurisdictions, where patient injury compensation claims are handled by the juridical system [12, 23]. Both these systems have their pros and cons, but one major advantage of the no-fault system is that it generates novel patient safety data for research and learning [40].

The most obvious and major limitation to this study is that we do not know the absolute numbers of each procedure performed in Scandinavia during the study period. Therefore, we cannot estimate the risk of compensation for the various surgical techniques. However, our study demonstrates the epidemiology of compensation claims and highlights the need of national cartilage registries. The study population was based on a set of predefined diagnosis and surgical procedures. Any kind of mislabeling of these by the orthopedic surgeon may cause some patients not to be included, introducing an inclusion bias. By using a broad range of diagnosis and surgical procedures and not only cartilage specific codes, we have tried to reduce this error. The total number of study subjects are relatively low, and may affect the results of this study.

The Scandinavian registries do not comprise all complications encountered after cartilage surgery. Some patients might have suffered a complication that would have led to compensation, but never filed a complaint to the registries. These factors may contribute to different biases to the cases available in the databases. The demographics do not include information such as ethnicity, socioeconomic status and insurance status, factors that we would like to illuminate.

Patient expectation management is important following cartilage restoration surgery. Our study is the first national report on compensation claims after cartilage injury and has focused on compensation claims after surgical treatment of focal cartilage defects in the knee. Knowledge of compensation claims following conservative treatment is lacking and should be highlighted in the future in the work on patient safety. Our study has demonstrated that the claim rate is low following these injuries and should be assessed in future research by validating patient's compensation claims by comparing institutional data with the filed compensation claims. Little is known whether health care professionals fail to inform patients of the possibility to file a compensation claim following a treatment injury. This topic should be addressed in future research.

Conclusions

Compensation claims following cartilage surgery in the knee are rare, and may suggest a lack of patient information on compensation claims from health care professionals. Establishing nationwide cartilage registries can add further knowledge on this troublesome disease.

Abbreviations

ACI: Autologous chondrocyte implantation; FCDs: Focal cartilage defects; ICD-10: International Statistical Classification of Diseases and Related Health Problems 10th Revision; MF: Microfracture; NCSP: NOMESCO Classification of Surgical Procedures; NPE: Norwegian System of Patient Injury Compensation

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Authors' contribution

TFA performed literature search, drafted and edited the article. OBL and LE gave critical review of the manuscript. AA launched the hypothesis of the study with study design and gave critical review of the manuscript and provided funding. PHR co-drafted and co-edited the article and launched the hypothesis and study design. All authors made contributions to design, was involved in the drafting and read and approved the final manuscript.

Authors' information
All authors are members of the Norwegian Cartilage Project, a Norwegian research organization focusing on improving the treatment of injured articular cartilage through five studies.

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Availability of data and materials
This study brought together existing data obtained upon request and subject to license restrictions from the National Swedish Patient Insurance Company, the Danish Patient Insurance Association and the Norwegian System of Patient Injury Compensation. The authors declare that the data supporting the findings of this study are available within the article.

Ethics approval and consent to participate
The study was conducted according to the World Association Declaration of Helsinki and was approved by the data protection officer of Helse Møre and Romsdal HF, Kristiansund Hospital, Norway (study no 2018/1357–11). As all data were based on already anonymized records, approval from the regional ethical committee was deemed not necessary.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Brief Report

Evaluating plasma extracellular vesicle microRNAs as possible biomarkers for osteoarthritis

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SUMMARY

Objective: MicroRNAs (miRNAs) are being launched as biomarkers for various diseases, but a robust biomarker for articular cartilage pathology has yet to be discovered. Here we evaluate plasma extracellular vesicle (EV) miRNAs as possible biomarkers for osteoarthritis (OA).
Method: We compared miRNA levels found in plasma EVs from patients with OA with controls without OA using next generation sequencing (NGS) technique. The patient and control pairs were matched for age, gender and body mass index.
Results: 23 pairs of patients and controls were included. Patients with OA differed significantly from controls in both clinical and radiological assessment of OA. We identified 177 canonical miRNAs in plasma EVs, but found no difference in miRNA levels between the two groups. Interestingly, the concentration of each miRNA in plasma EVs showed minimal difference between the participants, suggesting that the release of miRNAs in EVs from cells within the various organs is a tightly controlled process.
Conclusion: This is the first study using NGS in search of a miRNA biomarker in plasma EVs in OA. The levels of each plasma EVs miRNA were surprisingly similar for all participants. No plasma EVs miRNA can be used as a biomarker for OA.

1. Introduction

To find a biochemical marker indicating persistent articular cartilage pathology is of paramount interest. The marker may identify people with increased risk of developing pathology and allow the monitoring of disease progression, enabling earlier treatment to reduce symptoms and prevent the development of osteoarthritis (OA) [1]. Further, the biomarker may enable better evaluation of treatment effects.

Since the discovery of small, non-coding double-stranded RNAs 25 years ago, microRNAs (miRNAs) have been investigated as possible biomarkers of disease [2]. MiRNAs are predominantly found in the cell cytoplasm, but are also released as stable molecules bound to proteins, lipoproteins or contained in extracellular vesicles (EVs). EVs are found in most body fluids, are thought to function as intercellular communication packages and are easily accessible for analysis [3].

MiRNAs have been reported as potential biomarkers for cancer and cardiovascular disease [3].

A few studies have also shown differential expression (DE) of miRNAs in serum or plasma between patients with OA and controls [4–7]. The findings from these studies are inconsistent, but miR-885-5p was

Abbreviations: DE, Differential Expression; EVs, Extracellular vesicles; miRNAs, MicroRNAs; miR, MicroRNA; NGS, Next Generation Sequencing; OA, Osteoarthritis; TMM, Trimmed mean of M-values.

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upregulated in OA patients in two studies [5,7]. However, to date no single miRNA or group of miRNAs has been accepted as robust biomarkers for OA. Plasma EVs are intercellular information packages, and their miRNA content has been shown to be different from miRNAs carried in serum or plasma [8,9]. We hypothesized that plasma EV miRNA levels might act as biomarkers for OA. To test this hypothesis, we compared miRNA levels of plasma EVs between OA patients and matched controls using next generation sequencing (NGS) technique. To the best of our knowledge, this is the first study comparing miRNAs from plasma EVs in persons with and without OA.

2. Methods

2.1. Participants

23 patients with OA and 23 controls without OA aged 42–72 years were recruited from the Musculoskeletal pain in Ullensaker Study (MUST) [10]. All participants underwent comprehensive clinical examination by trained physicians, x-rays of both knees, hips and hands and blood samples. The groups were paired and matched by age, sex and body mass index. All patients had either radiographic OA (Kellgren-Lawrence grade 2 or more) in hip(s) and/or knee(s) (n = 21) or previous joint prosthesis of one hip (n = 1) or one knee (n = 1). The controls did not fulfil the American College of Rheumatology criteria for hands, hips and knees, had no prosthesis in hips or knees, had no radiographic OA in their hips or knees (Kellgren-Lawrence grade 0). Further, the controls had maximum two hand joints with mild radiographic OA (Kellgren-Lawrence grade 2) and no or doubtful OA (Kellgren-Lawrence grade 0–1) in remaining hand joints. The evaluation of hip, knee and hand radiographs were done by trained readers with good to excellent reliability.

2.2. Informed consent and ethical approval

Prior to inclusion in the MUST study, all participants signed a written informed consent which stated that blood samples would be stored in a biobank for future analysis of associations between clinical characteristics and biomarkers. The procedures followed were in accordance with the ethical standards of the Norwegian Regional Ethics Committee (Ref. no: 2009/812a and 2009/1703a) and with the Helsinki Declaration of 1975, as revised in 2000.

2.3. Collection of plasma and storage

4 mL of whole blood was obtained from each participant. 500 μL of plasma was collected in EDTA tubes and stored at – 80° Celsius. Qiagen services were used for EV isolation and miRNA sequencing (Qiagen, Vedbaek, Denmark).

2.4. EV size measurement

EVs were isolated by centrifugation of plasma at 16 000×*g* for 5 min before purification using the exoRNeasy Serum Plasma Kit according to the protocol from the manufacturer (Qiagen). EVs size distribution was performed using the Zetasizer Nano ZS system according to the manual from the manufacturer (Malvern Panalytical, Malvern UK).

2.5. Western blotting

EVs from 4 mL plasma were isolated and eluted in 200 μl Buffer XE (Qiagen). 200 μl of the eluate was mixed with 200 μl of 2x Laemmli Sample Buffer (Sigma-Aldrich, St. Louis, MO), vortexed for 20 s and incubated at 98 °C for 10 min to denature proteins. 35 μl lysate was loaded onto a 4–20% gradient polyacrylamide gel (Biorad, Hercules, CA). Proteins were separated by gel electrophoresis, transferred to PVDF membranes using the TransBlot Turbo system (Biorad) and incubated with rabbit anti-human ALIX, rabbit anti-human TSG101 and rabbit anti-human CD81 antibodies (Abcam, Cambridge, UK). After washing, incubation with a horseradish peroxidase-conjugated horse anti-rabbit IgG (H + L) secondary antibody (Vector labs, Burlingame, CA) and a final washing step the bands were visualized using the myECL imager (Thermo Fisher Scientific, Waltham, MA). All antibodies were diluted in 1X TBS, 5% nonfat dry milk, 0.1% Tween 20.

2.6. Library preparation and NGS

The library preparation was done using the QIAseq miRNA Library Kit (Qiagen). A number of spike-ins were added to the samples prior to RNA isolation. A total of 6 μL total RNA was converted into miRNA NGS libraries. Adapters containing unique molecular identifiers were ligated to the RNA before conversion to cDNA. The cDNA was amplified using PCR (22 cycles) and during the PCR indices were added. After PCR, the samples were purified. Library preparation quality control was performed using either Bioanalyzer 2100 (Agilent, Santa Clara, CA) or TapeStation 4200 (Agilent). All 46 samples formed adequate cDNA libraries and were sequenced. Based on quality of the inserts and the concentration measurements the libraries were pooled in equimolar ratios. The library pools were quantified using qPCR and sequenced on a NextSeq500 sequencing instrument according to the manufacturer instructions to a depth of 22 million reads per sample (average) with a single-end read of 51 nucleotides. Raw data were de-multiplexed and FASTQ files for each sample were generated using the bcl2fastq software (Illumina Inc., San Diego, CA). FASTQ data were checked using the FastQC tool. The RNA libraries construction and sequencing were performed on the Illumina platform with the NEBNext Multiplex Small RNA Library Prep Set for Ilumina (Illimina). All deep sequencing analyses were blinded, as those who prepared the samples and performed the data analysis were unaware of group affiliation. The study complied with the Minimum Information about a Microarray Experiment (MIAME) checklist. The workflow of the experiment is demonstrated in Fig. 1a.

2.7. Statistical analysis

Mean, median and standard deviation were calculated for continuous variables. Categorical data were presented in frequencies and cumulative frequencies. The reads mapping to mature miRNAs sequences were normalised using trimmed mean of M-values (TMM) normalization and edgeR was used for DE analysis of paired samples. Filtering on sequencing depth normalised values was applied before accounting for composition bias. A threshold of 12 counts per million reads in at least half the number of samples was used, as this corresponds to 5 reads in the smallest sample which can be seen as detection boundary.

3. Results

16 females and 7 males with similar age (58.0 and 57.7) and BMI (27.6 and 27.1) were included in the two groups. The clinical and radiologic evaluations were significantly different between the two groups. Demographics and clinical characteristics of the study population are presented in Table 1.

A schematic representation of the experiments is shown in Fig. 1a. The average size of the EVs was 235 nm (range: 70–900 nm) (Fig. 1b). ALIX, TSG101 and CD81, generally found in EVs, were all present in the EVs (Fig. 1c).

A total of 177 canonical miRNAs were detected in plasma EVs using our filtering criteria (Supplementary Table S1). However, a paired DE analysis did not reveal statistically significant differences in plasma EV miRNA levels between patients with OA and controls. A plot of twelve miRNAs known to be associated with OA [2] confirmed very similar miRNA levels in the two groups (Fig. 2a). The top 20 miRNAs for all 46 participants combined are plotted in Fig. 2b. They have all previously been detected in plasma EVs in other studies [11].

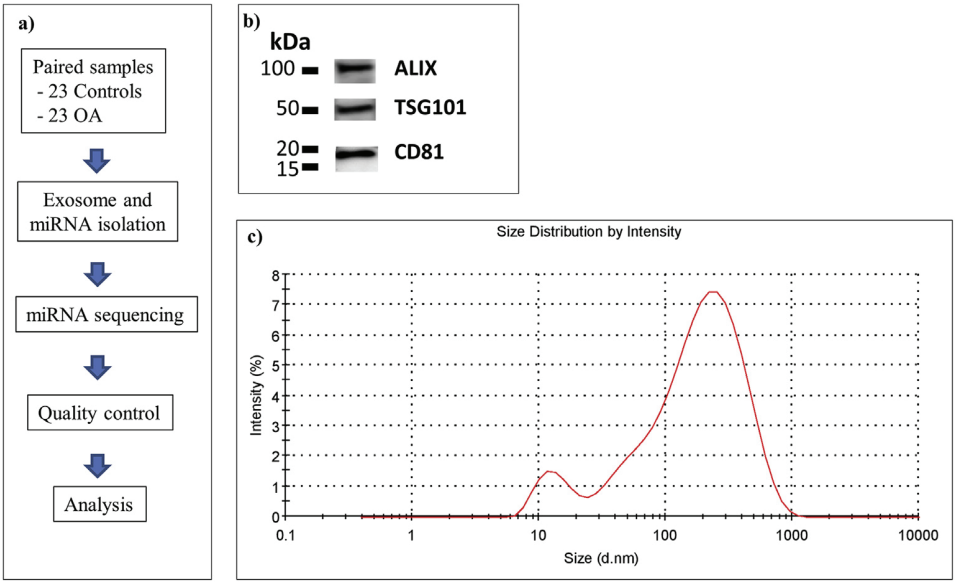

Fig. 1. a) Schematic overview of the experiments. b) Western blot of ALIX, TSG10 and CD81. c) Size distribution of the EVs. d - diameter, nm - nanometer.

Table 1
Demographic information about the study population.

	Persons with OA	Persons without OA	p-value
Sex, n (%) women	16 (69.6)	16 (69.6)	1.0
Age, mean (SD)	58.0 (7.0)	57.7 (7.3)	0.97
BMI, mean (SD)	27.6 (3.6)	27.1 (3.6)	0.91
Fulfil ACR criteria, n (%)			
Hip	3 (13.0)	0 (0)	0.23
Knee	19 (82.6)	0 (0)	<0.001
Hand	11 (47.8)	0 (0)	<0.001
K-L sum scores (median, range)			
Hip	1 (0–3)	0 (0)	0.49
Knee	2 (0–4)	0 (0)	<0.001
Hand	NA	1 (0–2)	NA

BMI – body mass index, ACR – American College of Rheumatology clinical evaluation, K-L – Kellgren-Lawrence radiologic evaluation, OA – osteoarthritis, NA – not assessed.

4. Discussion

The most important finding of the present study was that miRNA plasma EV levels from patients with OA were not statistically different from controls using NGS technique. We identified 177 miRNAs in plasma EVs. All of the 20 most abundant miRNAs have been detected in plasma EVs in other studies [11,12], lending support to our observations.

So far, reports of miRNAs as a biomarker for OA have mainly been based on studies of animals, human chondrocytes and synovial fluid [13]. Although miRNA studies are rapidly increasing, the results are inconsistent. miR-16-5p has been shown to be expressed at higher levels in OA cartilage than in healthy cartilage, and was hypothesized to control development of OA [14]. miR-16-5p was the most abundant miRNA in this study, but we found no difference between the two groups (Fig. 2b). Lin et al. found miR-30d to be highly expressed in human articular chondrocytes [15], whereas Withrow and colleagues demonstrated that miR-200c is elevated in synovial fluid in OA patients compared with non-OA controls [16]. We identified both miR-30d and miR-200c, but no statistically significant differences were detected between persons with OA and without OA (Fig. 2a). miR-140 has previously been found to play a significant role in OA pathogenesis and is hailed as a potential biomarker for articular cartilage pathologies [2]. miR-140 was detected in our material (Fig. 2a), but was not among the 20 most abundant miRNAs (Fig. 2b).

MiRNAs are predominantly found in the cell cytoplasm, but are also reported in extracellular fluids bound to proteins, lipoproteins or contained in vesicles such as EVs. The role of circulating miRNAs as potential biomarkers for OA is relatively unexplored. To our knowledge, there are only four studies comparing levels of circulating miRNAs between OA patients and controls [4–7]. Murata et al. compared concentrations of five different miRNAs in plasma and synovial fluid in patients with rheumatoid arthritis, knee OA and healthy controls [4]. They found that miR-16 and miR-132 were significant lower in OA patients compared with healthy controls, and stated that miR-132 could detect individuals with OA with 84% sensitivity. Beyer and colleagues analysed pooled serum samples from 13 OA patients who underwent hip or knee arthroplasty with pooled serum samples from 13 individuals without arthroplasty [5]. Using a microarray screen, they identified 12 miRNAs with DE. They then compared the levels of these miRNAs in 67 individuals with knee/hip arthroplasty with 749 individuals without, and identified three potentially predictive miRNAs for severe OA, namely let-7e, miR-454 and miR-885-5p. Ntoumou et al. used a microarray platform to compare serum miRNAs DE in 12 OA patients undergoing knee replacement surgery with 12 patients undergoing knee fracture repair surgery [6]. They found a significant downregulation of miR-33b-3p, miR-140-3p and miR-671-3p in OA serum compared to controls. Finally, Cuadra and associates compared circulating miRNAs in plasma from patients with primary knee OA with controls without clinical or radiological knee OA, also using a microarray platform [7]. They identified 12 elevated miRNAs in the OA group. As none of these studies investigated all possible miRNA sequences by NGS technology, and their results are largely non-overlapping, their usefulness as biomarkers of early OA remains to be proven.

The relative concentrations of miRNAs secreted in EVs are different from those found in the cell cytosol, and different from serum/plasma concentrations of miRNAs [17]. As EVs are thought to have a special role as intercellular messengers, we hypothesized that miRNA information about OA might be found in plasma EVs. In order not to introduce an a priori selection bias on our screening assay, we used NGS which picks up all possible miRNA sequences. The vast majority of the miRNAs found in the serum/plasma studies mentioned above and suggested to have a role

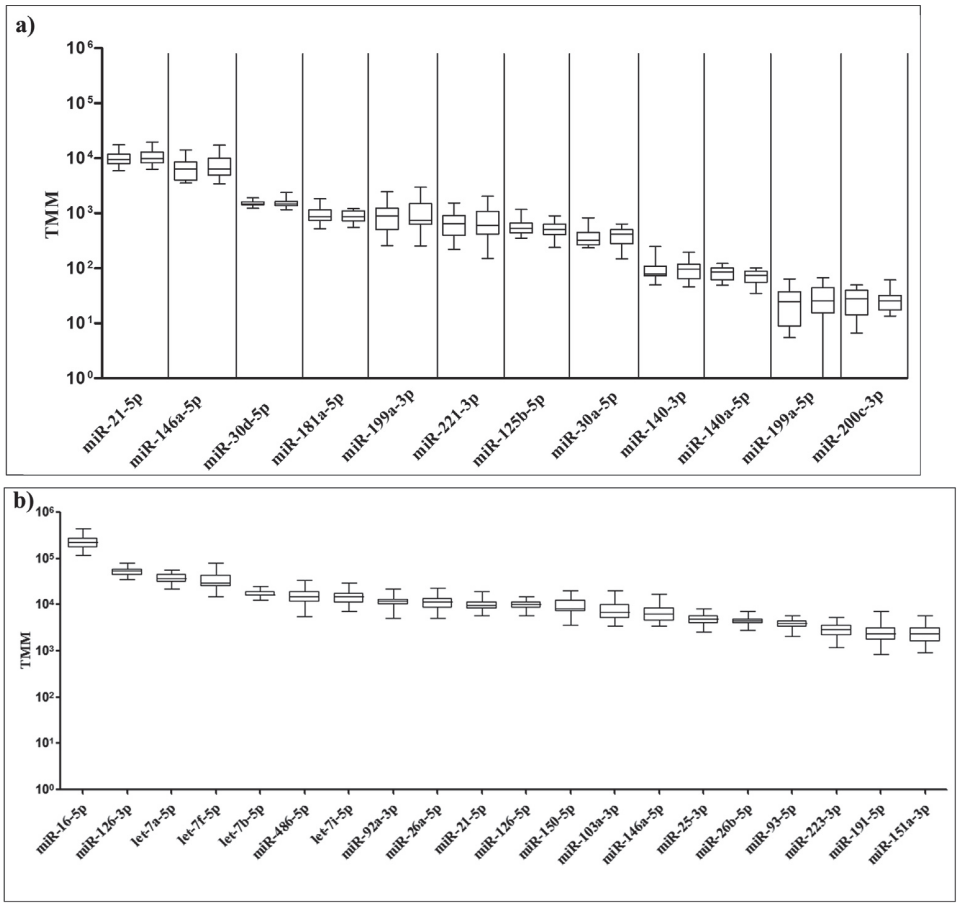

Fig. 2. a) Boxplots of 12 miRNAs associated with osteoarthritis and cartilage in controls (left) and patients (right). b) Boxplots of the 20 most abundant miRNAs. Controls and patients are shown as one group since there was no difference between the two groups. MiRNA – microRNA, OA – osteoarthritis, TMM – trimmed Mean of M-values.

as biomarkers were also found in plasma EVs, but with similar levels for patients and controls. Further, no other miRNAs were found to differ between the two groups. As we have investigated all possible miRNA sequences in a fairly large group (23 pairs) of OA patients and controls matched for age, sex and body mass index, the possibility of finding a miRNA biomarker for OA in plasma EVs must now be almost absent.

Still, the study has limitations. The plasma samples used in this study were stored at −80° Celsius for up to 7 years before analysis were performed. This may have affected our findings. Although the collection and handling of blood samples were performed after a strict study protocol, any inconsistencies in these procedures may alter the levels of miRNAs. However, the exoRNeasy Serum Plasma Kit has been compared with ultracentrifugation and other EV isolation kits, and found to be the best, indicating that no other isolation strategy would have more reliable results [18,19]. The yield of purified EVs in plasma is small (less than 0.1 μg of total RNA in 1 mL of plasma), and deep sequencing techniques usually requires at least 1 μg of total RNA. As we utilized only 500 μL of plasma, this may have led to greater variability among our results. However, the spike-in sequences were found at expected and reproducible levels, and the number of miRNAs identified was similar to those found in other studies [4], suggesting that our observations are likely to truly represent the relative concentrations of miRNAs in plasma EVs. Also, we do not have information about OA in other joints, such as hands (OA patients), spine, feet and shoulders, and cannot exclude that patients or controls had OA at these sites. This is a limitation affecting many biomarker studies. However, for the references used in this manuscript [4–7], at least we know that they had OA in the same joints (hips and/or knees) as the OA patients enrolled in the current study. Lastly, others have investigated long noncoding (lnc) RNAs in cartilage and found aberrant expression in OA [20]. The possible role played by lncRNA in the pathogenesis and as biomarkers of OA was not investigated in the present study, and remains to be fully determined.

As NGS has not yet been used in studies to identify circulating miRNA biomarkers for OA, there is still hope for this strategy. However, miRNAs are presumably released from all the cells in the body to eventually find their way into the blood stream, and miRNAs from cells of OA affected joints may just be too few to impact on the overall levels of circulating miRNAs. Indeed, the most remarkable observation presented here is the similarity in plasma EV miRNA levels between individuals, suggesting that this is a tightly controlled process.

5. Conclusion

This study is the first to compare circulating miRNAs in EVs in OA patients using NGS. We did not identify any plasma EV miRNAs that can potentially act as biomarkers for OA. Further research is necessary to identify a biomarker for early OA.

Contributions

TFA and TAK contributed equally and performed the literature search, interpreted the data, drafted and edited the article. IKH and MAR provided study material and gave critical review of the manuscript, ØBL gave critical review of the manuscript and provided funding and JEB launched the hypothesis of the study with study design, interpreted the data, gave critical review of the manuscript and provided funding. All

authors made contributions to conception and design, was involved in the drafting and read and approved the final manuscript.

Role of the funding source

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Data statement

The datasets generated and analysed during the current study are not publicly available due to the size of these datafiles but are available from the corresponding author on reasonable request.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ocarto.2019.100018.

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IV

SCIENTIFIC REPORTS
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Robust profiling of microRNAs and isomiRs in human plasma exosomes across 46 individuals

Tommy A. Karlsen[1*], Tommy F. Aae[2,3] & Jan E. Brinchmann[1,4]

microRNAs (miRNAs) are small double stranded RNA molecules consisting of two complementary strands called the 5p and 3p arms. Following imprecise processing and/or addition of nucleotides at the ends, miRNA biogenesis can give rise to variants called isomiRs. Exosomes are small vesicles released by cells. They have attracted attention due to their potential use in biomarker development because of their content of biomolecules, including miRNAs and isomiRs. Exosomes are found in body fluids such as plasma. In this study we used next generation sequencing to investigate the distribution of 5p and 3p arms of both miRNAs and isomiRs in plasma exosomes from 46 individuals. Among the canonical miRNAs there was similar prevalence between 5p and 3p miRNAs. Most of the miRNAs had isomiRs, and in approximately half of the cases an isomiR was more abundant than the corresponding canonical miRNA. Most of the isomiRs were generated from 5p miRNAs. There were very small differences in the concentration of canonical miRNA and isomiR sequences between donors, suggesting tight control of isomiR generation and sorting into exosomes. IsomiRs are abundant in plasma exosomes and should be included in analysis when plasma exosomal miRNAs are investigated as potential biomarkers for disease development.

MicroRNAs (miRNAs) are small double-stranded RNA molecules that regulate gene expression. They are involved in most, if not all, biological processes and have been found to be dysregulated in several diseases[1,2]. Monitoring miRNA levels in different cell types, tissues and body fluids, such as plasma, serum and urine has therefore attracted attention because of their potential use as biomarkers for disease development[3].

During their multistep biogenesis miRNAs are first transcribed as primary transcripts (pri-miRNA). These are processed in the nucleus by the Microprocessor complex, made up by the RNase III enzyme Drosha and two DGCR8 proteins, creating shorter precursor miRNA (pre-miRNA) molecules. The pre-miRNAs are then transported into the cytoplasm where they are further processed, by the RNase III enzyme Dicer, into mature double-stranded miRNA sequences with a length of approximately 20–22 nucleotides (nt). The mature miRNA consists of two sequences, the miRNA-5p (5p) and miRNA-3p (3p) strands, held together by base-pairing and with a 2 nt 3′overhang at each end[2]. After processing by Dicer, one or both of the strands are loaded into the Argonaute (AGO) protein. Here they bind to complementary mRNA molecules leading to either degradation of the mRNA or inhibition of translation[2]. AGO, with the miRNA, mRNA and several proteins mediating mRNA silencing or decay are collectively called the RNA-induced silencing complex (RISC).

miRNAs are defined by their unique sequences as listed in the miRBase data (http://www.mirbase.org/). These sequences are called canonical miRNAs and are defined by the consensus sequence in the database as the most abundant reads obtained from all recorded next generation sequencing (NGS) analysis experiments[4,5]. NGS analysis has revealed several miRNA variations at the ends or within the mature miRNA sequence. These variants are called isomiRs and are thought to be a result of imprecise processing by the Microprosessor complex and/or Dicer or due to addition of nt by nucleotidyl transfereases at the ends of the miRNAs. Another process, RNA editing, can change nt within the miRNA sequence[2].

The so-called seed sequence, nt 2–8 at the 5′end, is thought to be the most important sequence for binding of the miRNAs to complementary target mRNA sequences, while the 3′end is thought to be important for

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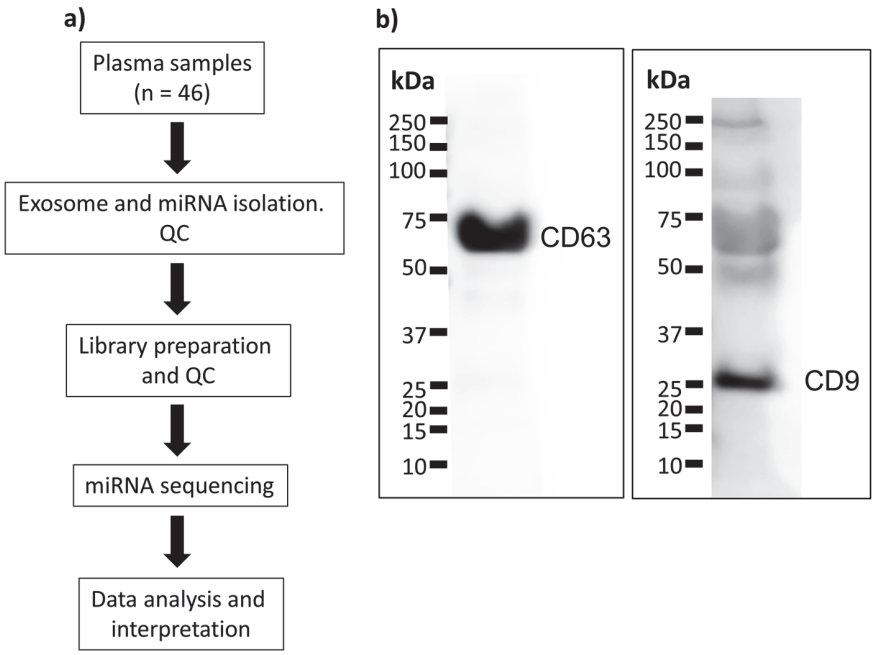

Figure 1. Set up. (**a**) Schematic diagram of the experiment. (**b**) Western blotting of CD63 and CD9 in plasma exosome lysate. The blots are from two different gels and cropped to fit into one figure. Different exposure times were used for the two blots. Unprocessed images of the blots are shown in Supplementary Fig. S1.

stabilization of the miRNA[2]. Thus, addition or deletion of nt at the 5′-end will shift the position of the seed sequence and give rise to a new seed with altered target specificity. Substitution of nt within the seed will also give rise to new seed sequences while changes at the 3′-end can affect the stability of the isomiR[2].

Exosomes are small (40–100 nm) extracellular vesicles that are released from multivesicular bodies (MVB) within the cells into the extracellular milieu. They are formed as intraluminal vesicles within endosomes and contain miRNAs and other non-coding RNAs, mRNA, DNA and proteins[6]. Sorting mechanisms that are not yet fully understood ensure that the exosomal miRNA cargo is different from the overall miRNA content of the parent cell. Exosomes are thought to be involved in intercellular communication since they can be delivered to other cells in the body where they release their content into the recipient cells. During disease the abundance of certain exosomal miRNAs can change[6]. Exosomes from body fluids, such as plasma and serum, has therefore attracted attention due to their potential role in diagnostics or as biomarkers of disease development. Several studies have analysed the content of miRNAs in plasma exosomes[6–8]. However, the distribution of 5p and 3p strands and their corresponding isomiRs in plasma exosomes is not sufficiently described and could be of importance in diagnostics.

In this study we have sequenced and characterized the content of canonical miRNAs and isomiRs in plasma exosomes from 46 individuals. 177 canonical miRNAs and 1716 isomiRs were detected. Both the 5p and 3p strands from the same miRNA were detected in 32% of the canonical miRNA sequences, only the 5p for 36% and only the 3p for 29% of the sequences. 67% of the canonical miRNA had isomiRs while 13% did not. The remaining 20% of the sequences were isomiR sequences where no belonging canonical sequences were detected. In 52% of the cases an isomiR was more abundant than the corresponding canonical miRNA. There was remarkably little difference in the concentration of canonical miRNAs and isomiRs between the donors, suggesting tight control both of the synthesis within the cells and of the release of these sequences into exosomes. IsomiRs are abundant in plasma exosomes and should be considered in biomarker analysis.

Results

A flowchart of the experiment is shown in Fig. 1a. Western blot of the tetraspanins CD63 and CD9, found in the membrane of exosomes, confirmed isolation of exosomes (Fig. 1b). An unprocessed image of the blot is shown in Supplementary Fig. S1.

Distribution of canonical 5p and 3p sequences. Supplementary Table S1 shows the names of all detected miRNAs and isomiRs, their sequences and TMM (Trimmed mean of M-values normalization) expression values for all samples. In total 1893 sequences were detected. Canonical miRNA sequences constituted 177 of these sequences while 1716 sequences were isomiRs. The abundance of canonical 5p and 3p sequences is shown in Fig. 2a. 32% of the canonical miRNAs sequences were 5p and 3p strands from the same miRNA (28 pairs), while for 36% and 29% of the miRNAs only the 5p or the 3p sequences was detected, respectively. Canonical miRNAs without a 5p or 3p annotation constituted 3% of the sequences. Figure 2b shows which of the two arms were the most prevalent among the 28 miRNAs where both strands were detected. 5p strands were more prevalent than

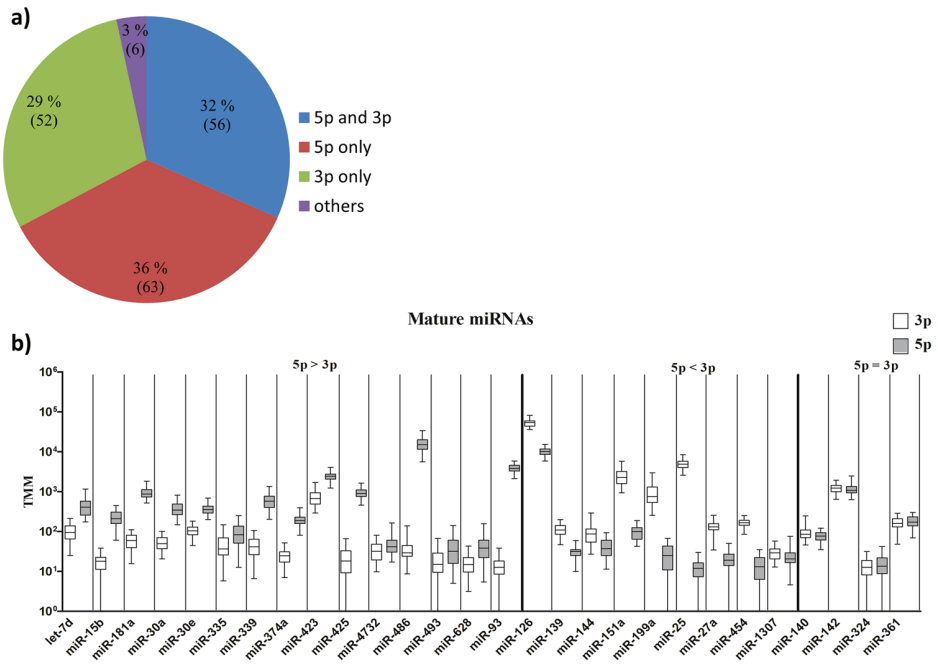

Figure 2. Distribution of 5p and 3p sequences. (**a**) Canonical miRNA sequences as deriving from 5p strands, 3p strands or from both strands of a miRNA. (**b**) Expression levels of canonical 5p and 3p among the 28 miRNA pairs where both strands were represented shown as boxplots with median, 75th% and 25th% percentiles and the minimum and maximum values. A paired t-test ($p = 0.05$) was used to test for differences. 5p = 3p represent non-significant results. TMM = Trimmed mean of M-values normalization.

3p in 54% of the pairs (15 of 28), 3p more prevalent than 5p in 32% of the pairs (9 of 28) and equal prevalence was detected in 14% of the pairs (4 of 28). Overall, canonical 3p sequences were found to be as numerous as 5p sequences in plasma exosomes.

Distribution of canonical and isomiR sequences. The number of exosome isomiRs per canonical miRNA varied hugely, from 0 to 103. The distribution of the sequences is shown in Fig. 3a. Canonical miRNAs without corresponding isomiR sequences constituted 13% of all sequences, while 67% of the sequences were miRNAs with corresponding isomiRs. IsomiRs without corresponding canonical miRNA sequences made up the remaining 20%. 26% of the isomiRs were variants of either strand of miRNAs where both 5p and 3p isomiRs were detected, 41% were from 5p sequences only, 27% were 3p sequences only and 6% were from miRNA without the 5p/3p annotation (Fig. 3b). In 52% of the cases at least one isomiR was more abundant than its corresponding canonical miRNA sequence (Fig. 3c). However, only 6.3% of the more abundant isomiRs contained new seed sequences. On the whole, many more isomiRs than canonical miRNAs were found in the exosomes and most isomiRs were from the 5p arm.

Stable miRNA and isomiRs concentration in plasma exosomes from different donors. The three most abundant sequences were miR-16-5p, miR-126-3p and a miR-142-3p isomiR. They are plotted, showing the 20 most abundant sequences, in Fig. 4a. miR-142-3p is an example where an isomiR sequence was more abundant than the canonical miRNA. There were surprisingly small inter-donor differences as shown by the very low 75–25 percentile boxes and the maximum and minimum values in the boxplots. Also for the least abundant sequences, such as miR-628-3p, the inter-donor differences were small for both canonical miRNAs and isomiRs (Supplementary Fig. S2).

New seed sequences in isomiRs. IsomiRs differ from canonical sequences at the 5′end, 3′end or within the sequence. Addition or removal of nt at the 5′-end and changes within the seed sequence will give rise to new seed sequences and therefore new mRNA targets, while changes at the 3′-end can affect stability[2]. The distribution of changes at the 5′end, within the seed and at the 3′-end varied a lot between different miRNAs. Figure 4b shows the fraction of isomiRs with changes leading to new seed sequences as well as changes at the 3′-end as a percentage of all isomiRs for the three most abundant miRNAs sequences shown in Fig. 4a, while Fig. 4c shows the fraction changes as the average percentages of all isomiRs found in plasma exosomes. On average 13% of all isomiRs contained new seed sequences, equally divided at 5.5% each between additions and deletions of nt at the 5′ end, with substitutions within the seed sequence found for approximately 2% of all isomiRs. However, when the TMM for sequences with new seeds was calculated as a percentage of the TMM for all identified sequences, most of these new seed sequences showed very low abundance. Table 1 shows the percentage concentration of new seed sequences among the 10 most abundant miRNAs measured as TMM relative to the TMM for all the isomiR

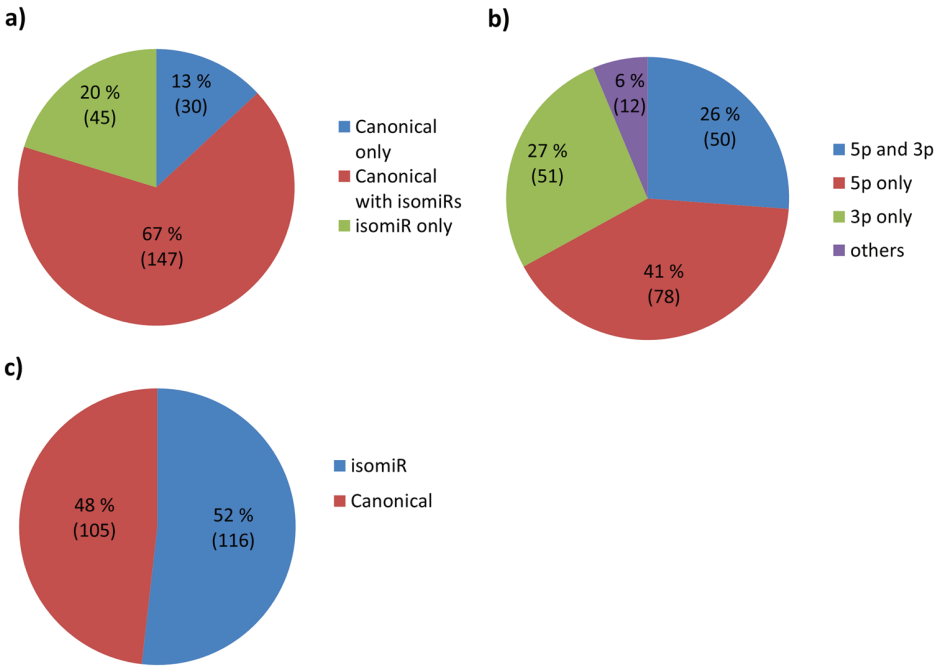

Figure 3. Canonical miRNA and isomiRs. (**a**) Distribution of canonical miRNA and isomiRs. Some miRNAs were represented by canonical sequences only (blue), some by both canonical sequences and isomiRs (red) and som by isomiR sequences only (green). (**b**) Distribution of isomiRs derived from 5p strands (red), 3p strands (green) or from both strands of a miRNA (blue). (**c**) Pie chart showing the proportion of miRNAs where the canonical sequence was most abundant (red) or an isomiR sequence was most abundant (red).

sequences within that miRNA. Except for miR-126-3p and miR-142-3p, isomiRs with new seed sequences always represented <2% of all sequences.

98.5% of all isomiRs had changes at the 3′ end. Loss of nt was more common than addition of nt at the 3′-end (Fig. 4c).

Using two target prediction databases, miRDB and Targetscan, to predict mRNA targets for the different miRNA sequences showed huge differences in the number of targets when comparing canonical miR-16-5p, miR126-3p and miR-142-3p with the most abundant of their isomiRs with new seed sequence (Fig. 5a–c). For the miR-16-5p isomiR 5 the new seed was a result of substitution of nucleotide number 5 (A/G). This resulted in a dramatic loss of predicted targets and only 3 targets were common between the canonical and the isomiR (Fig. 5a). For miR-126-3p the isomiR had more predicted targets than the canonical sequence, but only 1 and 2 targets were in common between the canonical and the isomiR in the two databases (Fig. 5b). Loss of one nucleotide at the 5′-end of miR-142-3p resulted in a new seed sequence that doubled the number of predicted targets and more than 60 targets were common between the canonical and the isomiR (Fig. 5c).

Discussion

Exosomes carry proteins and nucleic acids, including miRNAs and their isomiRs[6]. The RNA content within exosomes is protected against degradation by RNases and can therefore be isolated intact from body fluids[9]. Stability and availability make miRNAs and isomiRs promising as biomarkers. Although isomiRs have been studied in plasma and other tissues[10,11], to the best of our knowledge, no studies have investigated the abundance and distribution of isomiRs in plasma exosomes. The data in this study presents the distribution and abundance of canonical 5p and 3p arms and canonical miRNAs and isomiR sequences in plasma-derived exosomes.

It is not yet fully known how the selection of miRNAs for incorporation into exosomes occurs. One possibility is that miRNAs are transported by RNA-binding proteins (RBP) from the RISC to MVBs for exosome loading. However, it is also possible that the strand not incorporated into RISC is carried by RBP to the MVBs. One mechanism for sorting of miRNA sequences bound for exosomes is therefore at the level of binding to AGO. Which of the two strands, 5p or 3p, that are incorporated into the RISC complex depends on the thermodynamic stability of the strands and the identity of the 5′-terminal nucleotide[2]. The term "guide strand" has been used to name the active strand that is incorporated into RISC while "passenger strand" has been used to name the opposite and degraded strand[12]. Historically the 5p strand was thought to predominate as the guide strand[12]. However, while the correlation of their presence in exosomes and their binding to AGO is not yet known, the similarity in the prevalence of 5p and 3p strands in exosomes found here suggests that "guide" and "passenger" terminology for the 5′ and 3′ strands, respectively, is not helpful for our understanding of the mechanisms involved in the sorting of miRNAs bound for exosomes. Another sorting mechanism could be at the level of binding to RBP. This has already been shown for the RBP heterogeneous nuclear ribonucleoprotein A2B1, where both sequence and

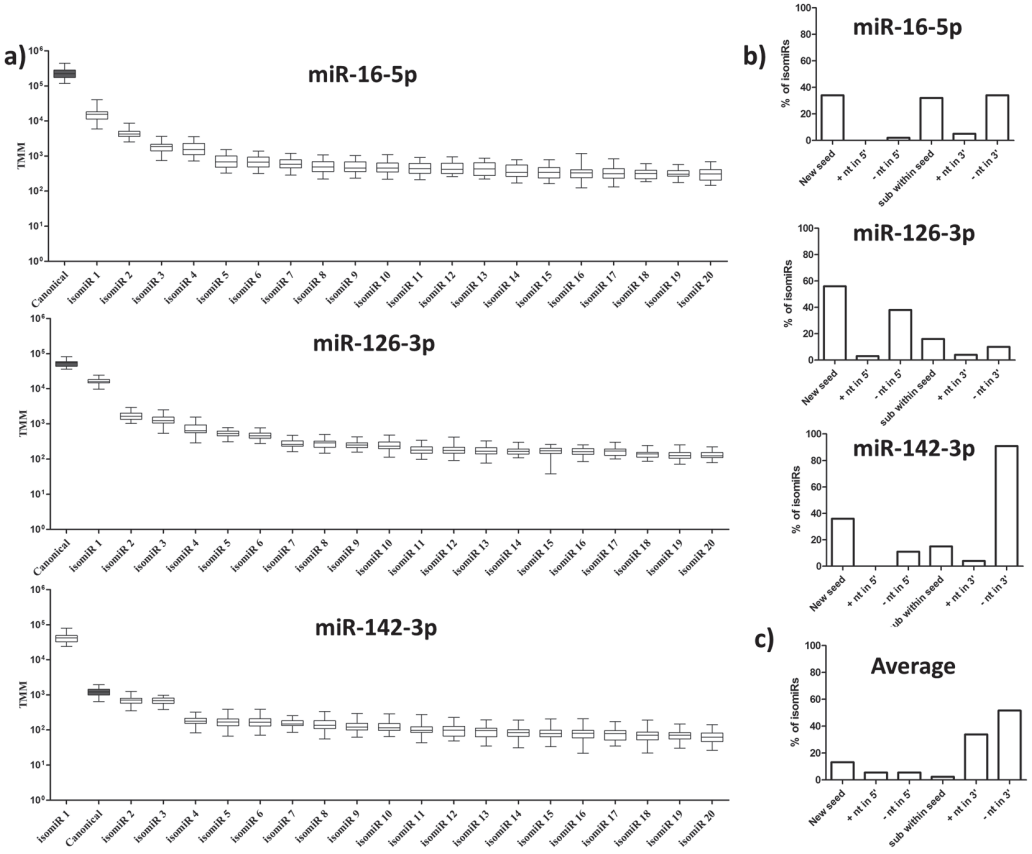

Figure 4. miRNA and isomiR expression. (**a**) Expression levels of canonical miR-16-5p, miR-126-3p, miR-142-3p (grey boxplots) and their top 20 isomiRs (white boxplots). TMM = Trimmed mean of M-values normalization. (**b**) Distribution of changes at the 5′-end, within the seed sequence and at the 3′-end of isomiRs for miR-16-5p, miR-126-3p and miR-142-3p. (**c**) Average distribution of changes at the 5′-end, within the seed sequence and at the 3′-end of all isomiRs. +nt in 5′ = addition of nucleotides at the 5′end, −nt in 5′ = removal of nucleotides at the 5′-end, sub within the seed = substitution of nucleotides within the seed sequence, +nt in 3′ = addition of nucleotides at the 3′end, −nt in 3′ = removal of nucleotides at the 3′-end.

	% new seed	% canonical seed
hsa-miR-16-5p	2.0	98.0
hsa-miR-126-3p	24.9	75.1
hsa-miR-142-3p	4.6	95.4
hsa-let-7a-5p	1.2	98.8
hsa-let-7f-5p	1.0	99.0
hsa-let-7b-5p	0.7	99.3
hsa-miR-486-5p	1.0	99.0
hsa-let-7i-5p	0.6	99.4
hsa-miR-451a	1.2	98.8
hsa-miR-92a-3p	1.2	98.8

Table 1. Percentage of average total TMM for top 10 expressed sequences.

sumoylation are determinants of miRNA binding[13]. Yet another factor affecting the prevalence of miRNAs in plasma exosomes is which cells are actually contributing miRNA-containing exosomes to the plasma pool. Here very little is known at the present time. Extreme possibilities are that each contributing cell type is responsible for all the copies of one or a few miRNAs, which is unlikely based on the heterogeneity found in miRNAs in exosomes derived for instance from synovial fluid or cell culture supernatants[14,15], or that every contributing cell type release exosomes containing most or all of the miRNA sequences described in this study. However, whichever mechanisms act in the regulation of miRNA sequences present in the plasma exosome cargo they seem to exert tight control, as suggested by the relatively low number of canonical miRNA sequences found – 177 out of a total of 1917 miRNAs found in miRbase – and the minimal difference in the prevalence of the different miRNA

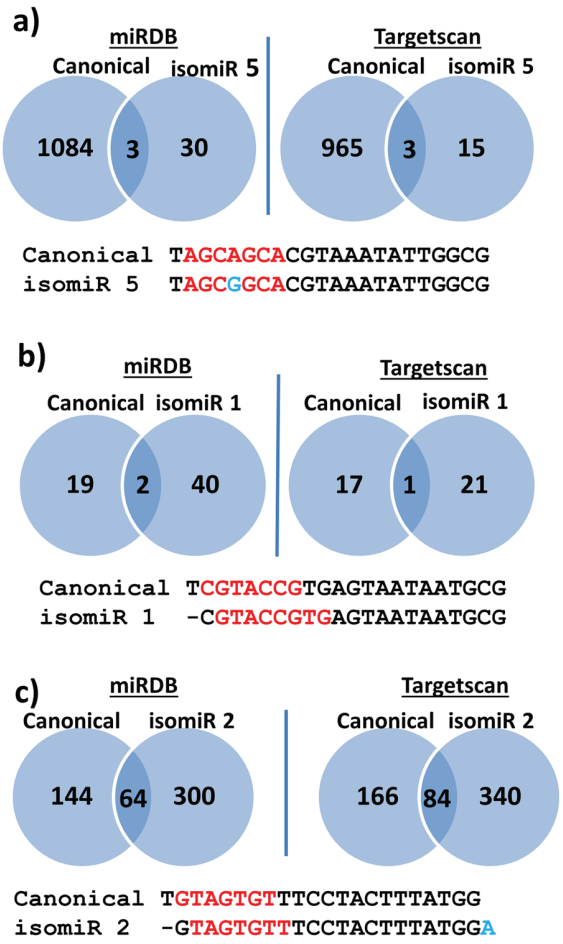

Figure 5. Target prediction. (**a**) mRNA target prediction of miR-16-5p and its isomiR 5 from Supplementary Table S1. The canonical miRNA and isomiR sequences are shown at the bottom with the seed in red and the nucleotide substitution in blue. (**b**) mRNA target prediction of miR-126-3p and its isomiR 1 from Supplementary Table S1 (**c**) mRNA target prediction of miR-142-3p and its isomiR 2 from Supplementary Table S1. The canonical and isomiR sequences are shown at the bottom with the seed in red and addition of a nucleotide at the 3′-end in blue. Numbers are the total number of mRNA targets predicted by each of the databases.

sequences observed between the 46 plasma donors studied here. Interestingly, canonical 3p miRNAs were as prevalent as 5p miRNAs in plasma exosomes, an observation which was also made following analysis of miRNA sequences in a very large number of human tissues[16].

The vast majority of canonical miRNAs found in plasma exosomes in this study were present together with one or more isomiRs. This is comparable with other studies on cancer cells and plasma[17–19]. In approximately half of the cases one isomiR, or several, was more prevalent than the corresponding canonical miRNA. This is similar to observations made in cultured chondrocytes, where only half of the top 20 expressed miRNAs had the canonical miRNA as the major expressed sequence[20]. Thus, isomiRs seem to be very common and highly expressed in many tissues and they probably have important roles in gene regulation. This is supported by studies were it has been demonstrated that isomiRs do indeed have a functional role[21]. isomiRs can have a different seed than its corresponding canonical miRNA and thus have different mRNA targets. New seed sequences were found in 13% of all the isomiR sequences. This was equally a result of removal and addition of nt at the 5′-end, with substitution within the seed occurring less commonly. In silico analysis showed that there were huge differences in the number of predicted targets and few shared genes between the canonical miRNAs and the isomiRs. However, it should be noted that isomiRs with new seeds made up a very small fraction, compared to sequences with the canonical seed,when taking expression level into account. The importance of plasma exosomes in cell communication is not well known, but *in vitro* and *in vivo* studies have shown that exosomes can be delivered and taken up by recipient cells and affect gene expression[6]. Thus, both for their roles in intercellular communication and also for their use as biomarkers for disease one would think, and hope, that changes in the miRNA cargo in exosomes released from cells in sick organs are sufficient in magnitude that distant cells, and investigating laboratories, will detect that change within the content of plasma exosomes. Based on current knowledge, that change may occur both

in canonical miRNA and in isomiR sequences. For profiling and functional studies it is therefore important to include isomiRs in the analysis.

PCR based assays, microarrays and northern blots do normally not discriminate between highly similar sequences. Consequently, many miRNA profiling studies have measured not only the canonical miRNAs, but also one or several of their isomiR sequences. As isomiRs are now known to be at least as prevalent as their canonical miRNAs, this suggest that miRNA quantification studies published using these assays may have based their conclusions on incorrect data. For functional studies, when cells are transfected with pre-made miRNA mimics, the sequences are based on the canonical miRNA sequence in miRbase. If the main functionality of the miRNA in question is exerted by an isomiR, these miRNA mimics are unlikely to reproduce that functionality. However, by using custom-made mimics it is possible to study the functionality of both canonical miRNA and their isomiRs. Transfection of plasmids or viral vectors with the miRNA gene, on the other hand, will presumably be processed by the Microprocessor complex and Dicer and thus give rise to isomiRs. However, whether the generated isomiRs are similar to the endogenous isomiR pool is unknown. This strategy may give correct information about the functionality of the processed pri/pre-miR, but not of the canonical miRNA or individual isomiR sequences.

We conclude that the release of miRNA and isomiRs into exosomes seems to be a tightly regulated process. Surprisingly the 3p strand was found to be as prevalent as the 5′ strand in plasma exosomes and both strands were associated with isomiRs. IsomiR analyses should be included when biomarker studies are being planned.

Methods and Materials

Collection of plasma and storage. Blood from 46 individuals was collected in EDTA tubes. The donors were recruited from the Musculoskeletal pain in Ullensaker Study (MUST)[22] as 23 patients with osteoarthritis (OA) (7 males and 16 females; mean age 58 years, range 45–72; mean body mass index 27.6, range 23.5–39.2) and their age, gender and body weight matched controls without OA (7 males and 16 females; mean age 57.7 years, range 42–72; mean body mass index 27.1, range 22.3–38.4) in a study to look for a miRNA biomarker for OA in plasma exosomes. However, a paired differential expression analysis, using a quasi-likelihood F-test, showed no difference between patients and controls in the expression of either canonical miRNAs or isomiRs after multiple hypothesis testing (false discovery rate was above 0.99 for all sequences). A paper describing patient characteristics, the expression of OA related canonical miRNAs and the 20 canonical miRNAs with the highest expression has been submitted for publication. In the present analysis of all canonical miRNAs and isomiRs found in plasma exosomes the patients and controls are analysed as one group, as no difference in expression was found between the diagnostic subgroups. All donors signed a written informed consent. The study, including all methods and experiments, was approved by the Regional Committee for Medical Research Ethics, Southern Norway, Section A. The study was performed in accordance with the ethical standards of the Helsinki Declaration of 1975, as revised in 2000.

After centrifugation the plasma was stored at −80° Celsius until exosome isolation and analysis. Exosome isolation and miRNA sequencing were performed using Qiagen services (Qiagen, Vedbaek, Denmark). Exosome miRNAs were isolated using the exoRNeasy Serum Plasma Kit according to the protocol from the manufacturer (Qiagen). This is a column based kit where exosomes are captured on a membrane and allows for removal of contaminants such as plasma proteins and protein/AGO-bound miRNAs[23].

Library preparation and NGS. The library preparation was done using the QIAseq miRNA Library Kit (Qiagen). Adapters containing unique molecular identifiers were ligated to the RNA before conversion to cDNA. After PCR (22 cycles), the samples were purified. Library preparation quality control was performed using either Bioanalyzer 2100 (Agilent, Santa Clara, California, United States) or TapeStation 4200 (Agilent). The libraries were pooled in equimolar ratios and sequenced on a NextSeq500 machine as single-end reads (51 nucleotides) with an average depth of 22 million reads per sample. FASTQ files were generated using the bcl2fastq software (Illumina Inc., San Diego, California, United States) and checked using the FastQC tool. The reads were mapped to the GRCh37 reference genome using Bowtie2 (2.2.2). Reads were normalised using trimmed mean of M-values (TMM) normalization. Filtering on sequencing depth normalized values was applied before accounting for composition bias. A threshold of 12 counts per million reads (CPMs) in at least half the number of samples was used for inclusion of miRNAs in the analysis.

IsomiRs were identified as follows: analysis was performed individually for each sample based on the occurrence of count variants for each detected microRNA. Reads were mapped to known microRNAs according to the annotation in miRBase and then investigated for the presence of different isomiRs. These variants were identified by changes in start or stop position, or occurrence of mutations within the read. The results for each sample were then merged to generate a single count file with a consistent nomenclature across the samples. Only isomirs that were present at a level of 5% of total reads for that miRNA were retained.

IsomiRs were identified as such, and not novel miRNAs, because they mapped to miRbase version 20 miRNA reads with less than perfect matching. Reads with variations to the miRbase reference, such as mismatch and alternative start/end positions, were reported as isomiR counts. Novel miRNAs would not map to miRbase reads, but would map to the genome at loci that do not encode known miRNAs.

The study complied with the Minimum Information about a Microarray Experiment (MIAME) checklist. The sequencing data are available from the corresponding author on reasonable request.

Western blotting. Exosomes were isolated using the exoRNeasy Serum Plasma Kit and eluted in 100 µl Buffer XE (Qiagen), mixed with 100 µl of 2x Laemmli Sample Buffer (Sigma-Aldrich, St. Louis, MO), vortexed for 10 seconds and incubated at 98 °C for 10 minutes to denature proteins. 35 µl lysate was loaded onto a 10% polyacrylamide gel (Biorad, Hercules, CA). Proteins were separated by gel electrophoresis, transferred to PVDF membranes using the TransBlot Turbo system (Biorad) and incubated with mouse anti-human CD9 (Abcam,

Cambridge, UK) and mouse anti-human CD63 (Abcam) antibodies. After washing, incubation with a horseradish peroxidase-conjugated horse anti-mouse IgG (H + L) secondary antibody (Vector labs, Burlingame, CA) and a final washing step the bands were visualized using the myECL imager (Thermo Fisher Scientific, Waltham, MA). All antibodies were diluted in 1X TBS, 5% nonfat dry milk, 0.1% Tween 20.

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Author contributions

All authors planned and conceived the study. J.E.B. supervised the project. T.F.A. was responsible for obtaining plasma samples from the MUST study. Most data analysis was conducted by T.A.K. with contributions by T.F.A. and J.E.B. T.A.K. wrote the original draft of the manuscript. All authors reviewed and approved the final manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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