UNIVERSITY OF LATVIA

FACULTY OF MEDICINE

FACTORS INFLUENCING BONE MARROW MONONUCLEAR CELL QUANTITY AND QUALITY IN PATIENTS WITH KNEE OSTEOARTHRITIS

DIPLOMA THESIS

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Valdis Gončars (Apr 30, 2020)

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Abstract

Thesis title: Factors influencing bone marrow mononuclear cell quantity and quality in patients with knee osteoarthritis.

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May 2020

Osteoarthritis is the most common joint disease with significant economic burden and increasing prevalence (Conaghan *et al.* 2014). One major problem is that there is no treatment, besides join replacement, which can cure osteoarthritis, that is why it is important to investigate alternative treatment methods such as stem cell therapy. The interest towards the research of mononuclear cells as a treatment method is widely increasing but still only a little is known about the optimal quantity of mononuclear cells injected and why the mononuclear and mesenchymal stem cell quantity and quality differs between people.

Aims of the research is to see if there are any correlations between patient factors, such as age, sex, erythrocytes, hemoglobin and thrombocytes, and the quantity of mononuclear cells, $CD34^+$ cells and the percentage of $CD34^+$ cells.

Gončars *et al*. (2018) did a great research using autologous bone marrow-derived mononuclear cells as a treatment method for knee osteoarthritis. They observed a wide yield of extracted mononuclear cells ($8.3 \times 10^6 – 158 \times 10^6$) even though the same harvesting and cell processing method was used. Patient's database of 33 people was used to find out factors which could be related to this wide range of cell yield. Some volunteers were also included originally in the study of Jakobsons *et al*. (2018) "Tissue evacuated during joint replacement as a source of mononuclear cells".

In my study, no significant correlations between the investigated patient's factors (age, sex, erythrocytes, hemoglobin, platelets) and the quantity of mononuclear cells, $CD34^+$ cells and percentage of $CD34^+$ cells were found. The only significant correlation was between the quantity of $CD34^+$ cells and mononuclear cells.

Keywords: knee osteoarthritis, mononuclear cells, stem cells, bone marrow, articular cartilage

Kopsavilkums

Diplomadarba nosaukums: Kaulu smadzenu mononukleāro šūnu daudzumu un kvalitāti ietekmējošie faktori cela locītavas osteoartrīta pacientiem.

Diplomadarba autors: Janne Juhani Viiala

Diplodarba vadītājs: pasniedzējs Valdis Gončars, Dr.med, PhD

Maijs 2020

Osteoartrīts ir visizplatītākā locītavu slimība ar ievērojamām ekonomiskām izmaksām un pieaugošu izplatību (Conaghan *et al*. 2014). Viena no galvenajām problēmām ir tā, ka nav nekādas ārstēšanas, izņemot locītavas aizvietošanu, kas var izārstēt osteoartrītu, tāpēc ir svarīgi izpētīt alternatīvas ārstēšanas metodes, piemēram, cilmes šūnu terapiju. Interese par mononukleāro šūnu izpēti kā ārstēšanas metodi ir plaši pieaugusi, bet vēl tikai nedaudz ir zināms par optimālo ievadīto mononukleāro šūnu daudzumu un par to, kāpēc mononukleāro un mezenhimālo cilmes šūnu daudzums un kvalitāte cilvēkiem ir atšķirīga.

Pētījuma mērķis ir noskaidrot, vai starp pacientu faktoriem, piemēram, vecumu, dzimumu, eritrocītiem, hemoglobīnu un trombocītiem, un mononukleāro šūnu, CD34 + šūnu daudzumu un CD34 + šūnu procentuālo daudzumu, pastāv kāda korelācija.

Gončars *et al*. (2018) veica lielisku pētījumu, izmantojot autologās, kaulu smadzenēs iegūtas mononukleārās šūnas, kā ārstēšanas metodi ceļgala osteoartrītam. Viņi novēroja lielu ekstrahēto mononukleāro šūnu daudzumu (8,3 x 106 – 158 x 106), lai gan izmantoja to pašu savākšanas un šūnu apstrādes metodi. Pacientu datubāze ar 33 cilvēkiem tika izmantota, lai noskaidrotu faktorus, kas varētu būt saistīti ar šo plašo šūnu ražības diapazonu. Daži brīvprātīgie sākotnēji tika iekļauti pētījumā Jakobsons *et al*. (2018).

Manā pētījumā netika konstatētas būtiskas korelācijas starp izpētītajiem pacienta faktoriem (vecums, dzimums, eritrocīti, hemoglobīns, trombocīti) un mononukleāro šūnu, CD34 + šūnu un CD34 + šūnu procentuālo daudzumu. Vienīgā būtiskā korelācija bija starp CD34 + šūnu daudzumu un mononukleāro šūnu daudzumu.

Atslēgvārds: ceļa locītavas osteoartrīts, mononukleārās šūnas, cilmes šūnas, kaulu smadzenes, artikulāra skrimšļa

Introduction

Osteoarthritis (OA) is a disease which disrupts everyday life of numerous individuals around the world. Conghan *et al*. (2014) evaluates that over 40 million people in Europe have osteoarthritis. Any joint can be affected, but knee, hip, spine and hand are most commonly affected by OA. The American association of orthopedic surgery estimates that the incidence of OA is about 240 per 100 000 people in the USA. According to a Finnish study the prevalence of knee OA in male is 5% and in women 7% (Terveys 2000).

New treatment modalities are needed for the treatment osteoarthritis. Incidence of OA is increasing due to ageing population and increasing obesity. The use of analgesics is a symptomatic treatment, but it is not always enough, and a joint replacement may be needed. Intra-articular corticosteroids are commonly used but they only treat the inflammation and they have a variety of side-effects if used prolonged time. One possible future treatment method is the use of mesenchymal stem cells which have the ability to promote the development of healthy tissue. There are different opinions whether these mesenchymal stem cells are just signaling cells which cause the differentiation of local tissue specific stem cell or do they differentiate directly into damaged tissue. These cells can be found almost everywhere, most commonly they are aspirated from the bone marrow as a part of mononuclear cells or separated from adipose tissue.

There is still a lack of knowledge of the optimal quantity of cells to be used and the factors affecting the quality and quantity of these cells. These are hot topics in the field of mononuclear cell therapy. Some factors are proven to influence the quality and quantity of MNC. These are contamination with erythrocytes (Assums *et al.* 2010), apoptotic cell content (Monquet *et al.* 2011), different steps in washing and centrifugation speed (van Beem RT *et al.* 2008). Marrow Conversion Index calculated from proximal femur MRI had a correlation with the quantity of mononuclear cells and mesenchymal stem cells in Suh *et al*. (2012) study.

Erythrocyte contamination and erythroid precursors can reduce the quality and quantity according to Assums *et al.* (2010) and Jaatinen (2007). Age is a factor that influences the neovascularization and was first described by Edelberg (2002). Aging shortens the telomeres, causing genomic instability and activation of p53 causing cell cycle arrest and reduced number of hematopoietic stem cells. Yong Sang Kim *et al.* (2015) found that clinical failure of MSC therapy was significantly influenced by age over 60 years and the cartilage lesion over 6.0 cm^2 significantly

influenced the clinical outcome in a negative way if compared to lesion size less than 6.0 cm^2. Dimmeler *et al.* (2008) states that patients with type I and type II diabetes mellitus (DM) have a smaller number of hematopoietic stem cells, defined as the $CD34^+$ cells and endothelial progenitor cells.

Jo *et al.* (2014) did a study of intra-articular injection of different quantities of adipose tissue derived mesenchymal stem cells for the treatment of knee osteoarthritis. They found that high-dose group (10.0×10^7 cells) improved in functionality of the knee measured with Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) six months after injection and reduced pain was observed with Visual Analog Scale and Knee Society Clinical Rating System (KSS) score, and without any adverse effects.

The goal of my study is to investigate patient's factors that could influence the quantity or quality of mononuclear cells and $CD34^+$ cells which were harvested from the bone marrow of a selected group of people or from the excised tissue harvested during joint replacement. The goal of the study was also evaluation of the laboratory method and its potential effect to the quantity and quality of the mononuclear cells.

The hypothesis of my study is that patient factors, such as age, sex, erythrocytes, thrombocytes and hemoglobin have correlation with the quantity of mononuclear cells and $CD34^+$ cells extracted from the bone marrow or harvested from excised tissue during joint replacement.

Literature Review

1. Epidemiology, risk factors and cost of osteoarthritis

Knee osteoarthritis (OA) can be found in 5% of men and 7% of women, whereas hip OA numbers are 5% in men and 4% in women (Terveys 2000). The prevalence of knee OA is shown in figure 1. The incidence of knee OA in the United States is 240 per 100 000 per year and it led to over 11 million ambulatory care visits in 2009 and approximately 10 million adults had symptomatic knee OA. The American Association of Orthopedic Surgery also states that the prevalence of knee OA is somewhere between 6-13% in men and between 7-19% in women (AAOS 2015).

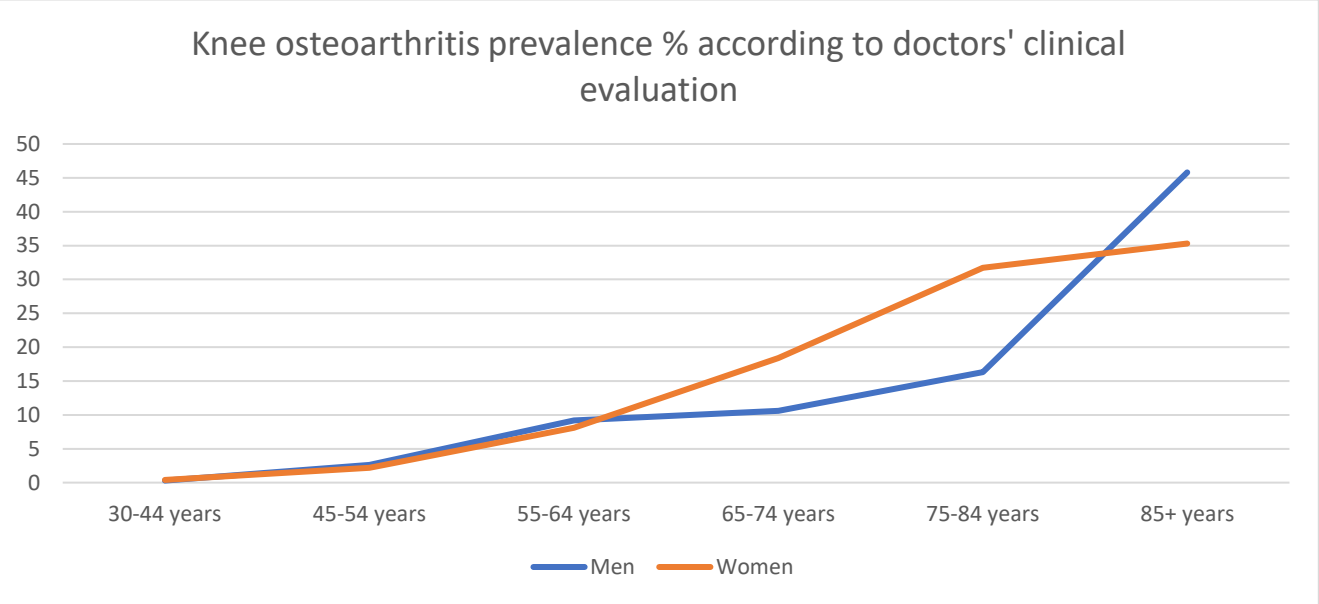

Figure 1 - Knee OA prevalence % according to doctors' clinical evaluation (Terveys 2000).

Well known risk factors of OA which cannot be influenced are increased age, female gender and genetics. Other risk factors that can be influenced are increased body mass, occupational load and traumas (AAOS 2015). These risk factors are not the same in all the joints. For example, sport is a higher risk factor in knee OA as well as overweight than in hip or hand OA. Joint deformity is a higher risk factor in hip OA than in knee or hand OA (Arokoski *et al.* 2008).

Osteoarthritis was the second most expensive condition in 2013 treated in the United States hospitals with hospital cost over 16,5 billion dollars covering over 4% of the national cost. The number of hospital-stays in 2013 were over one million (2,9%). Only septicemia was more expensive condition. (Celeste 2013)

2. Pathogenesis of OA

Series of biochemical reactions induced by abnormal mechanical load or pathological cartilage tissue in the joint will lead to OA. OA will have changes, not only in the cartilage, but also in subchondral bone tissue, synovium and muscles. In molecular level, OA consists of degenerative and regenerative reactions. (Arokoski *et al.* 2008)

The first stage of OA is characterized by increased quantity of water due to break down of collagen fibers IX and XI. Type II collagen fibers do not change in the beginning. Proteoglycan aggregation and quantity decreases in the first stage. The thickness of cartilage can slightly increase during this stage, due to increased synthetic activity of chondrocytes. After this stage the surface collagen fibers start to break down even more and proteoglycans are lost from the surface of cartilage. Subchondral bone can also become thicker. The ability of chondrocytes to synthesize more collagen fibers and proteoglycans is lost due to decreased sensitivity to anabolic factors and direct mechanical force. This causes the cartilage to become thinner and finally is lost completely. This causes increased bone synthesis in subchondral area. Please see figure 3. (Arokoski *et al.* 2008)

Radiological imaging shows cysts, resorption of the bone and generation of osteophytes which limits the movement of the joint. The articular capsule gets thicker. The damaged or completely lost cartilage tissue cannot regenerate anymore. The radiological finding of OA can be divided according to Kellegren and Lawren into 0 to IV stages, where stage 0 is a normal knee joint and stage IV is a significantly narrowed joint space with large osteophytes, bony deformity and severe sclerosis. Stage I criteria are doubtful narrowing of the joint space and possible osteophyte lipping. Stage II has already definite osteophytes but still doubtful narrowing of joint space. Stage III is defined as definite joint space narrowing, sclerosis and multiple large osteophytes with possible bony deformity. (Arokoski *et al.* 2008)

Arokoski *et al.* (2008) describes how different factors influence the metabolism of articular cartilage in a molecular level. The basic idea is that matrix metalloproteases (MMP) break down the cartilage extracellular matrix. MMPs can be activated by different inflammatory factors, such as IL-1, TNF-α and nitric oxide (NO). Anabolic factors TGFβ, insulin like growth factor-1 (IGF-1) and bone morphogenic proteins (BMP) cause increased production of proteoglycans, but unfortunately nitric oxide decreased the production of these anabolic factors.

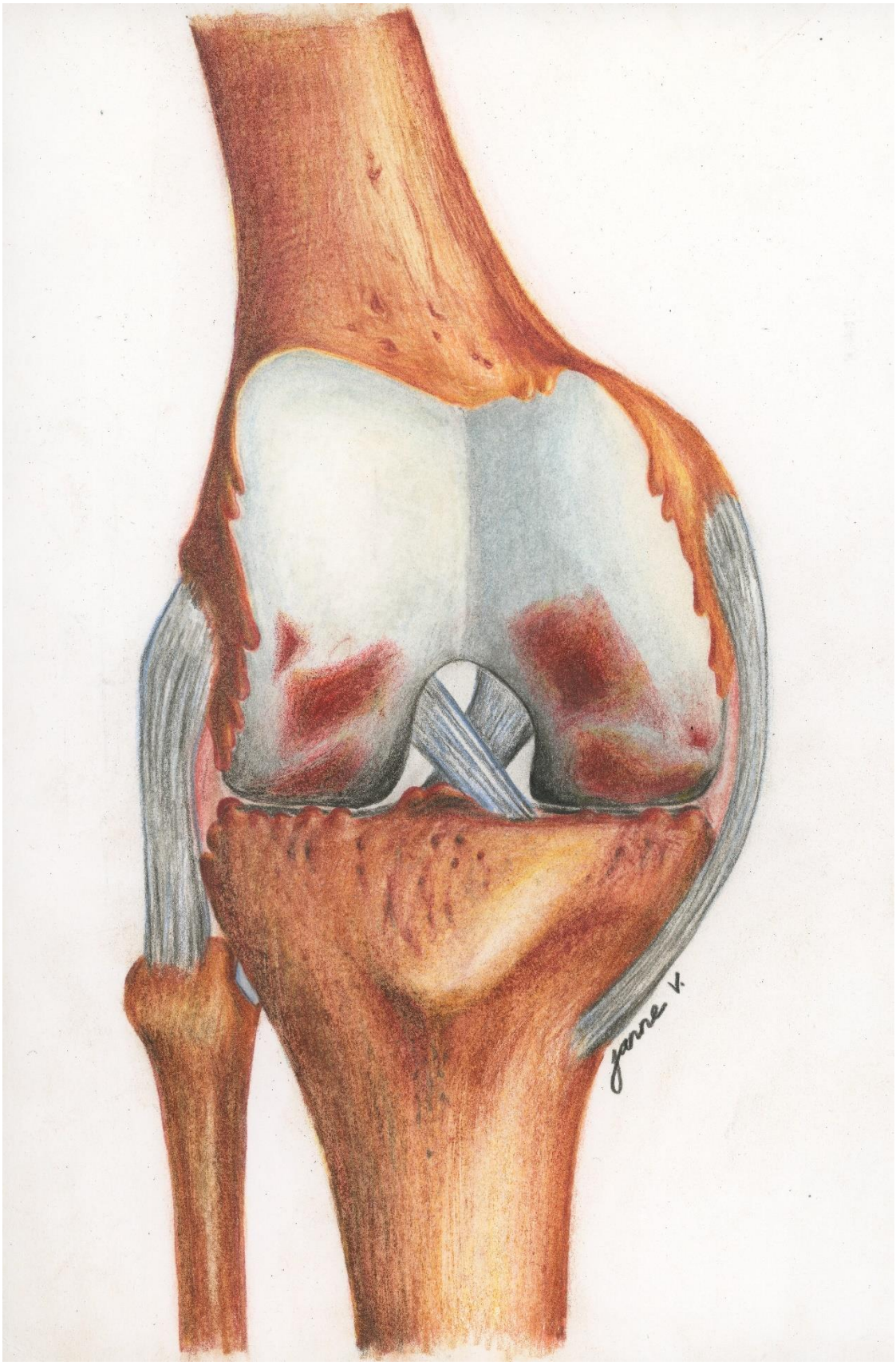

Figure 2 - Knee osteoarthritis

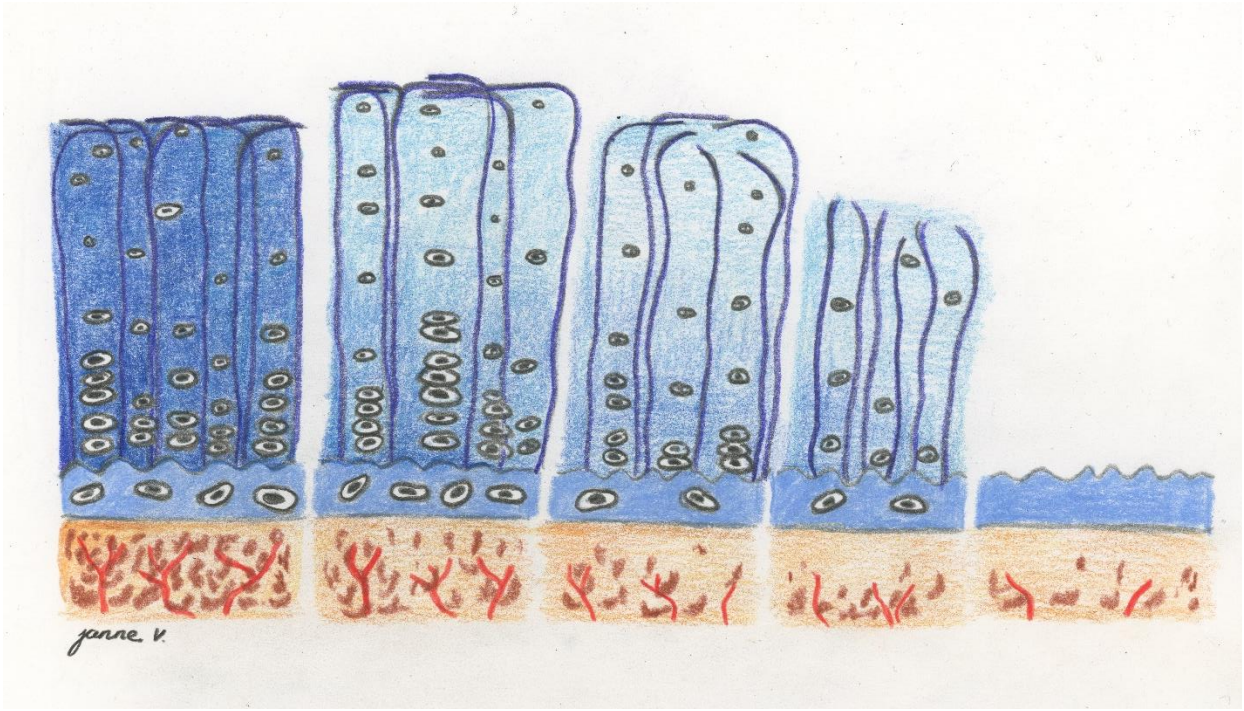

Figure 3 - Articular cartilage, stages of osteoarthritis

3. Current treatment of knee OA

Current Care Guidelines of knee OA, published in 2018 by The Finnish Medical Society Duodecim, recommends that the treatment should be individual with indications and contraindications taken into account, but the main goals of the treatment is pain control, improve the functionality and prevent the disease progression. There are no curative treatment methods available at the present time.

Conservative non-pharmacological treatment methods are the basis of the treatment. These include weight loss if one is obese by combining nutritional change and regular physical exercise which can be instructed by physiotherapist. Good methods of exercise are walking, cycling, swimming and specific exercises for the muscles supporting the knee. Compressive or twisting exercises are not recommended. Physical therapy methods include cryotherapy, surface heat therapy and Transcutaneous Electrical Nerve Stimulation (TENS) -therapy. Knee orthosis or elastic supports can be used to reduce the pain and increase functionality. Aids, like claw grabber or walking canes can be used if needed. (Current Care Guidelines 2018)

Arokoski *et al.* (2008) writes that the first line pharmacological treatment is Paracetamol with maximum dose three grams a day, usually given one gram three times per day. Paracetamol is safer drug than NSAIDs, because it does not affect the COX-2 or COX-1 enzymes and synthesis of

prostanoids and thus does not cause problems with gastrointestinal tract or blood coagulation. NSAIDs are given if the paracetamol is not enough. NSAIDs should be used as short time as possible with the lowest possible dose, preferably in 7 to 21 day course. Use of NSAIDs increases the risk of gastric bleeding and ulcus, thus proton pump inhibitors are recommended to use simultaneously to protect the gastric mucosa. NSAIDs are also contraindicated in patients with cardiovascular diseases. New selective COX-2 inhibitors are safer to use due to less gastrointestinal side-effects. Local NSAID gels are safe to use alone or with other medication. If combination of paracetamol and NSAIDs are not enough, then opioids, for example tramadol or codeine, can be used. They have more side-effects, like nausea and constipation and can cause dependence.

Arokoski *et al.* (2008) and Current care Guidelines (2018) talk about the intra-articular injections to treat the knee OA. Glucocorticoid injections suppress the inflammation and have analgesic effect with a duration from weeks to months depending on the individuals. Unfortunately, intra-articular glucocorticoid injections have possible side-effects, from which bacterial arthritis is the most severe. Intra-articular glucocorticoid injections also increase the damage of cartilage, so the use of them should be limited to once per month with the maximum of 3-4 times in one year (Zhang *et al.* 2008). The method of injection is important to know. Current care guidelines (2018) instructs that 2ml of methylprednisolone with lidocaine 40/10mg ml solution, which gives 80mg of methylprednisolone is injected into the knee joint using 20-24 G sterile needle, sterile gloves and sterile drapes from the lateral side of the knee between the upper and middle third of the patella, after cleaning the injection area, while pressing the patella from the medial side causing the lateral side to open up a bit. Sterile plaster is placed over the injection area. Cold may be applied to reduce the pain and the joint should not be used excessively during the first day. Lidocaine analgesic effect will fade away after a few days causing increased pain for a short period of time. Injection should never be pressed against a force.

Hyaluronate injections are another option to glucocorticoid injection (Arokoski *et al.* 2008). They are commonly used if glucocorticoid injections are contra-indicated or not tolerated. Hyaluronate is a macromolecule, a polysaccharide which is made of N-acetyl-D-glucosamine and D-glucuronic acid disaccharide units. Hyaluronic acid is synthesized in the joint by synovial cells and chondrocytes. Proteoglycans, which are attached to hyaluronate for a brush-like structure giving the joint its strength against pressing force. Three to five hyaluronate injections are given one injection per week.

Some patients benefit from glucosamine medication. Glucosamines are found in glycosaminoglycans of the articular cartilage. Glycosaminoglycans are polysaccharide chains consisting of uronic acid combined with glucosamine or galactosamine. Theoretically glucosamines can increase the synthesis of proteoglycans, decrease the synthesis of proteoglycan degrading substances like metalloproteinases and aggrecanases and decrease inflammatory mediators like IL-1, nitric oxide and prostaglandins. The studies about the effectiveness are controversial and oral glucosamine medication increases the glucosamine concentration only a little in the joint. If the patient does not benefit from glucosamines after one to three months, the drug should be discontinued. (Arokoski *et al.* 2008)

Joint replacement surgery improves the quality of life and can be considered as the choice of treatment method if pain and functional ability do not improve with conservative treatment in addition to radiological progression of the OA. At the present moment, there are no treatment methods which would cure the OA. (Arokoski *et al.* 2008)

3.2 Stem cells

Arnold I. Caplan, Skeletal research Center in the Department of Biology of Case Western Reserve University, Ohio, started to study in the late 1960s with a chicken limb bud the ability of the mesodermal cells to induce chondrogenesis. In 1995 the first clinical trial using MCSs was done by Lazarus HM *et al.* (1995). They collected 10ml of bone marrow from 23 patients and separated the mononuclear cells. Autologous mesenchymal progenitor cells were reinfused intravenously, and bone marrow was examined two weeks later giving them result that no adverse reactions were observed.

3.2.1. Types of stem cells

Dimmeler *et al.* (2008) states that there are two types of stem cells. Embryonic stem cells, which have the capacity to differentiate into various types of different cells, tissues and organs. The other type of stem cells are adult stem cells, which differentiation is more limited. There are different types of adult stem cells, including bone marrow derived stem cells, circulating pool of stem cells and tissue-resident stem cells. Bone-marrow derived stem cells are a group of stem cells, containing hematopoietic stem cells, mesenchymal stem cells, multipotent adult progenitor cells and side population cells.

International Society of Cell Therapy defines the MSC to be positive for stromal cell markers such as CD73, CD105, CD90 and negative for hematopoietic markers such as CD45, CD34, CD14,

DC19, CD11b and HLA-DR (Dominici 2006). Other requirements for MSC are that they should be plastic-adherent when maintained in standard culture condition and that they must be able to differentiate to adipocytes, chondroblasts and osteoblasts in vitro. Dimmeler *et al.* (2008) describes that important hematopoietic stem cell markers are $CD133^+$, $CD34^+$ and vascular endothelial growth factor -receptor 2, also known as KDR or flk-1.

The way how mesenchymal stem cells differentiate into chondrocytes is a challenging and complex pathway consisting of multiple different factors such as transcription factors sox-9, runx-2, TGF-β3 and many other modulators and proteins (Gupta 2012). Quality of cartilage produced by bone marrow derived stem cells is different than the chondrocytes themselves produce. Kiviranta *et al.* (2012) writes that synovial and periosteum mesenchymal cells can differentiate into chondrocytes, but they tend to form fibrocartilage, which is not ideal type of cartilage for joint. Fibrocartilage does not have the typical collagen fiber arrangement which is seen in hyaline cartilage in normal joints. Fibrocartilage consists of type I collagen, whereas hyaline cartilage consists of type II, IX and XI collagens.

Arnold. I. Caplan, one could call him as the father of mesenchymal stem cell studies, writes in the Stem Cell Journal (2017) that it is time to change the name of mesenchymal stem cells, which were named 25 years ago. Caplan described already in 2010 that mesenchymal stem cells are known to secrete different bioactive factors and they have regenerative and immunomodulatory properties and thus should be named as Medicinal Signaling Cells. According to Caplan, MSC are not stem cells and they should not be called as stem cells. They, with the present understanding, do not function as progenitor cells. According to Caplan, if mesenchymal stem cells are infused on the site of injury, they secrete bioactive factors which will cause site-specific resident stem cells to construct new tissue.

3.2.2. Sources of mesenchymal stem cells

According to Kiviranta *et al.* (2012) mesenchymal stem cells (MSC), also called incorrectly as mesenchymal stromal cells by some scientists, can be isolated from bone marrow, adipose tissue, muscles, deciduous teeth, synovium, periosteum, cancellous bone, articular cartilage, tendon, pericytes, dermis, umbilical cord and placenta. These sources have different quantity of MSC per tissue weight. Adipose tissue has greater number of MSC per tissue weight than bone marrow aspiration according to Kiviranta *et al.* (2012). This makes the adipose tissue an interesting target of investigation, especially because adipose tissue is easily accessible.

Caplan *et al.* (2017) summarizes that MSC could possibly be derived from pericytes which are wrapped around endothelium. These pericytes are located throughout the whole body. MSC can thus be isolated from any vascularized tissue. It could be possible that when injury occurs, pericytes are activated and they differentiate into MSC, which will secrete bioactive factors causing tissue- and site-specific stem cells to differentiate into a new tissue. These tissue-specific stem cells are located near the pericyte MSC.

Fraser *et al.* (2006) discussed that only a few hundred of MSC can be found from one milliliter of aspirated marrow. The number of MSC according to Fraser *et al.* is 500 times greater in adipose tissue. They measured the quantity by detecting colony forming units (CFU-F and CFU-AP). More than 40 years ago, Friedenstein *at al.* (1974) demonstrate the ability of specific bone marrow cells to proliferate and adhere to plastic, these cells had spindle-like appearance and thus they were named as colony forming unit fibroblasts (CFU-F).

Jakobsons *et al.* (2018) did a great study where they compared two different sources of mononuclear cells; *crista iliaca* puncture and excised tissue which was removed during cleaning of the joint operation site including bone marrow, peripheral blood and fat. Mononuclear cells from both groups were isolated by Ficoll density gradient centrifugation and flow cytometry was used to detect the mononuclear cells, CD34+ cells and cell viability. They found that 34 *crista iliaca* punctures gave on average $28.64 \pm 9.35 \times 10^6$ MNCs and $0.77 \pm 1.51 \times 10^6$ CD34+ cells. Excised tissue gave on average $76.67 \pm 35.42 \times 10^6$ MNCs and $1.33 \pm 0.97 \times 10^6$ CD34+ cells, which is 2.7 times higher compared to the *crista iliaca* puncture. One must note that the puncture gave only 46.31 ± 9.35 ml of bone marrow solution and excised tissue gave 450 ± 157.69 ml of tissue solution.

4. Methods of cell-harvesting and problems related to the process

Pösel *et al.* (2012) discusses that multiple different methods of BM MNC harvesting, from which most studies choose Ficoll-Paque density medium for centrifugation to enrich the MNC population. Some factors are proven to influence the quality and quantity of MNC. These are contamination with erythrocytes (Assums *et al.* 2010), apoptotic cell content (Monquet *et al.* 2011), different steps in washing and centrifugation speed (van Beem RT *et al.* 2008). Some other factors have been discussed as well, which could maybe influence the final cell yield and possibly the outcome of the treatment. These factors are the depth of the aspiration needle inserted, the thickness of the iliac

crest, negative pressure, amount of aspiration punctures, used anticoagulant, peripheral blood volume in the sample and the diameter of the needle (Jakobsons *at al.* 2018).

4.1. Ficoll-Paque density gradient centrifugation

Ficoll-Paque density gradient centrifugation medium is one of the most commonly used density gradient medium in studies to enrich the mononuclear cell population (Pösel *et al.* 2012). In Ficoll-Paque centrifugation medium anticoagulant-treated and diluted bone marrow biopsy material or some other blood material like umbilical cord blood is carefully layered on the Ficoll-Paque solution which has been adjusted ideally to the temperature of 18 to 20 degrees of Celsius. This medium is then centrifugated. Centrifugation will cause the movement of erythrocytes and granulocytes into the bottom of the tube. The next layer from the bottom is the Ficoll-Paque media. Monocytes, platelets and some lower density lymphocytes are placed in a layer between the Ficoll-Paque layer and the first layer consisting of plasma (Jaatinen 2007), this layer is the mononuclear cell layer. At high temperature, around 37 degrees of Celsius, aggregation of erythrocytes and mononuclear cells are increased. At low temperature, around 4 degrees of Celsius, the aggregation is decreased but separation time increases which causes worse mononuclear cell separation. Density of Ficoll-Paque media decreases if the temperature increases (GE Healthcare Bio-Sciences AB 2014).

Mononuclear cells, i.e. lymphocytes and monocytes are not dense enough to penetrate the Ficoll media and that is why they can be found between the plasma and Ficoll media, but erythrocytes and granulocytes, which aggregate at room temperature with a high molecular synthetic polymer PM400 found in Ficoll-Paque media causing them to sediment and move to the bottom of the tube. Within the mononuclear cell layer are the most important and interesting cells, the rare mononuclear stem cells (MSC). Mononuclear cells can be collected from the tube, first by removing the plasma layer above the mononuclear cells and then collecting mononuclear cells by a sterile pipette. Then they can be washed to remove the platelets, plasma and Ficoll-Paque left in the layer. There is also Ficoll-Paque Premium, which has a benefit that lymphocytes with a higher density than the media will move across the media causing even more enriched mononuclear cell layer with cells having lower density, like mesenchymal stem cells.

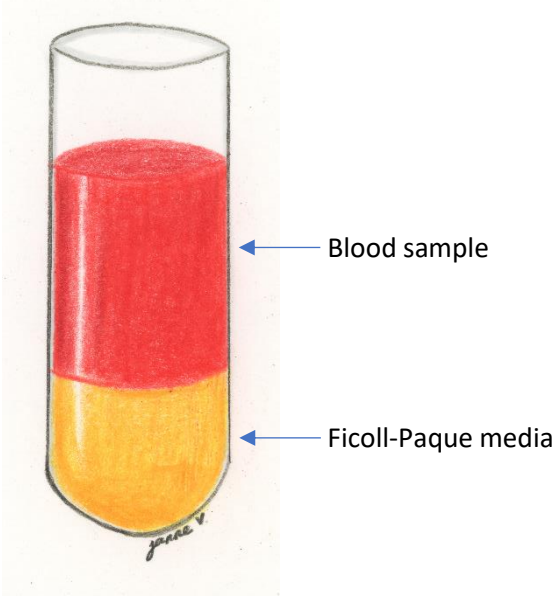

Figure 4 - Before centrifugation

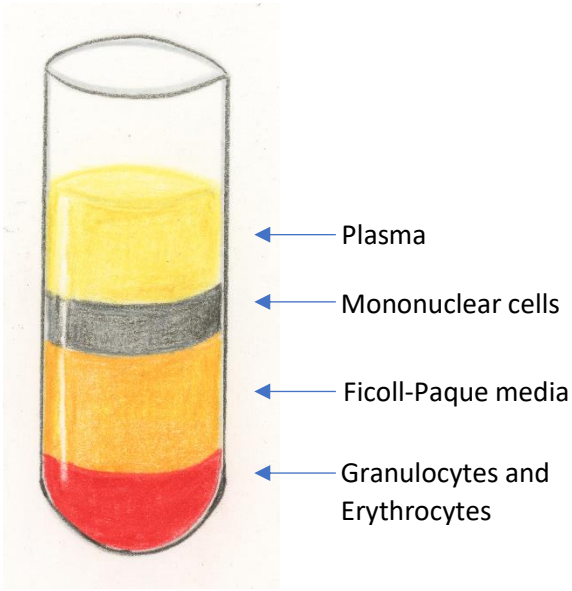

Figure 5 - After centrifugation

4.2. Contamination with erythrocytes

Assums *et al.* (2010) studied the quality and functional activity of bone-marrow derived progenitor cells used for patients with acute myocardial infarction to recover the contractility. They found that the left ventricle ejection fraction four months after intracoronary bone marrow mononuclear cell therapy was significantly reduced and reduction correlated with the contamination of the cell product by erythrocytes. This outcome was confirmed by experimental studies where erythrocytes were added to bone marrow mononuclear cells causing impaired function in vitro and in vivo.

Jaatinen (2007) describes the problem of erythroid cells. These cells do not sediment in the bottom layer as expected. Some nucleated erythroid progenitor cells may stay at the layer of mononuclear cells causing harm. Jaatinen also considers that erythrocytes may aggregate and adhere to lymphocytes causing sedimentation of lymphocytes in the bottom. This can be reduced by diluting the blood sample. Jaatinen recommends diluting the blood sample 1:4 by using a phosphate-buffered saline to reduce aggregation of erythrocytes. If the blood sample is cryopreserved before the centrifugation it may cause aggregation of cells due to damaging the cells during the process of melting the blood sample. If cryopreserved blood is used then a different anticoagulant citrate dextrose solution should be used to reduce aggregation even more.

Jaatinen writes that the blood volume and tube diameter are critical for successful isolation of pure mononuclear cells, because increasing the height of the blood sample increases the contamination with erythrocytes. Larger volume of blood can be centrifugated in a tube of larger diameter with constant centrifugation time without changing the result of purification. GE Healthcare Bio-Sciences AB (2014) keeps 2.4 cm of Ficoll-Paque media and 3.0 cm of blood sample as a standard.

4.3. Apoptotic cells

Injected bone marrow mononuclear cells may poorly survive locally. Monquet *et al.* (2011) did a study of the apoptosis rate in bone marrow mononuclear cells which were prepared for cardiac therapy. Cells in the BM MNC injection, which have engaged apoptotic process may not be documented properly by the routine viability tests. These apoptotic cells could release microparticles to induce the apoptosis of other cells nearby, thus impair the efficacy of the therapy (Monquet *et al.* 2011).

4.4. Washing of cells and centrifugation speed

Van Beem RT *et al.* (2008) investigated the recovery and functional activity of mononuclear bone marrow cells and peripheral blood cells after different washing protocols and different centrifugation speed used. They found that cell recovery was significantly lower in a processing protocol without human serum albumin and heparin in the washing buffer. They also found that centrifugation speed of 600 to 800 g gave significantly better cell recovery compared to speed of 250 g.

Jaatinen (2007) recommends a centrifugation speed of 400 g for 40 minutes without brake when separating mononuclear cells and when washing the mononuclear cells twice in 40ml of phosphate-buffered saline centrifugate should run with a speed of 300 g for 10 minutes with break. GE Healthcare Bio-Sciences AB (2014) recommends also centrifugation at 400 g for 30 to 40 minutes at room temperature without brake.

4.5. Angiogenetic capacity

Assums *et al.* (2007) have shown that colony-forming unit (CFU) activity of the bone marrow MNC correlates with angiogenetic capacity in patients with chronic ischemic heart disease. Neovascularization capacity was the measure of functional capacity of the BM MNC. They also described that cell-isolation procedures may affect the functionality of the BM MNC. Changes in temperature of the storage, buffer solution choice or use of plasma from the patients during the cell

processing and isolation impairs the CFU capacity, thus the functionality, capacity to migrate toward chemoattractant and angiogenesis in hindlimb ischemia patients (Assums *et al.* 2007).

Matoba *et al.* (2008) did a study about therapeutic angiogenesis by cell transplantation. They injected intramuscularly autologous bone marrow mononuclear cells for patients with chronic limb ischemia and found that the cell therapy is not inferior to the conventional revascularization. Tateno *et al.* (2006) reports that the use of peripheral mononuclear cells was effective for limb ischemia treatment and it was associated with increased angiogenic factor IL-1 beta in plasma. They think that the cell therapy stimulates muscles to produce angiogenic factors. Pösel *et al.* (2012) writes that the mixture of different cell population from aspirated bone marrow, including B and T lymphocytes, monocytes, hematopoietic stem cells, mesenchymal stromal cells, endothelial progenitor cells and very small embryonic-like cells stimulates the angiogenesis, but it is not understood yet how. Bhartiya *et al.* (2012) reports that very small embryonic-like cells (VSEL) are lost during bone marrow processing due to their small size. These pluripotent stem cells have the maximum regenerative potential. They also concluded that VESL are the true stem cells in adult body tissues and hematopoietic stem cells and mesenchymal stem cells are progenitor stem cells that arise by asymmetric cell division of VSEL.

5. Patient factors influencing the clinical outcome and proliferation capacity of progenitor cells

Yong Sang Kim *et al.* (2015) evaluated retrospectively 55 knees after mesenchymal stem cell implantation for knee OA with a Kellegren-Lawrence grade I or II, symptoms of joint pain and limitations of normal function after 3 months of conservative therapy. They harvested the mesenchymal stem cells by using buttocks liposuction. International Knee Documentation Committee, Tegner activity scale and overall satisfaction of the patients were used to evaluate the clinical outcome. They found that clinical failure of MSC therapy was significantly influenced by age over 60 years. In addition, they found that the cartilage lesion over 6.0 cm^2 significantly influenced the clinical outcome if compared to lesion size less than 6.0 cm^2. Other factors like sex, side of involvement, BMI and lesion location did not have significant effect on the clinical outcome. One must notice an important bias of the study, the number of the knees were small especially when dividing the patients into subgroups.

Age as a factor that influences the neovascularization was first described by Edelberg (2002). Aging shortens the telomeres, causing genomic instability and activation of p53 causing cell cycle

arrest and reduced number of hematopoietic stem cells. Choudhery *et al.* (2014) concluded in their study about human adipose derived MSCs that advanced age causes reduced proliferation capacity and viability of the MSCs, importantly, poor chondrogenic and osteogenic differentiation potential was significantly affected by advanced age.

Dimmeler *et al.* (2008) states that patients with type I and type II diabetes mellitus (DM) have a smaller quantity of hematopoietic stem cells, defined as the $CD34^+$ cells and endothelial progenitor cells. Animal experiences found similarly diminished function of the progenitor cells in obese diabetic mice, including impaired migration towards vascular endothelial growth factor and thus diminished neovascularization capacity (Awad *et al.* 2005). High glucose level in a diabetic person, according to Thum *et al.* (2005), reduces nitric oxide bioavailability and affect also the level of endogenous nitric oxide synthase inhibitors in plasma which with a complex mechanism with proinflammatory protein kinases and reactive oxygen species (ROS) affect the migratory effect of progenitor cells.

Loomans *et al*. (2004) also studied about the effect of type 1 DM to the endothelial progenitor cells (EPC) which were cultured from circulating mononuclear cells. They found 44% decrease of EPC in type 1 DM patient compared to non-diabetic control group. In addition, they found that increased HbA_{1C} causes decreased number of EPC. Fadini *et al.* (2005) found the same type of results from type 2 diabetic patients: the circulating EPC and circulating progenitor cells (CPC) were significantly less in type 2 diabetic patients than in non-diabetic control group.

6. How quantity of the cells influences the outcome in OA patients

One of the hot topics of research in the field of mesenchymal stem cells and mononuclear cell intra-articular injection is the quantity of cells which are injected. It is unknown what the optimal quantity is to achieve the best clinical outcome. Jo *et al.* (2014) did a study about intra-articular injection of adipose tissue derived mesenchymal stem cells for the treatment of knee osteoarthritis. They divided the doses into three different groups: low-dose consists of 1.0×10^7 cells, mid-dose consists of 5.0×10^7 cells and the high-dose consists of 10.0×10^7 cells. Three patients were included in each group in the first phase of the study, and in the second phase nine patients were included in the high-dose group. Total of 18 patients were investigated. The small number of participants is a bias in the study. High-dose group improved in functionality of the knee measured with Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) six months after

injection and reduced pain was observed with Visual Analog Scale and Knee Society Clinical Rating System (KSS) score, and without any adverse effects.

6.1. MRI as a tool to predict the quantity of mesenchymal stem cells

It would be great if we had the tool to predict the quantity of mesenchymal stem cells in a bone marrow aspirate. How many cells can we get with aspiration from the bone marrow may influence the outcome of the treatment. I found one study where the bone marrow mesenchymal stem cell quantity was measured and the correlation with MRI image of proximal femur metaphysis was investigated (Suh *et al.* 2012).

Investigators calculated so called Marrow Conversion Index (MCI) from MRI image by measuring the intensity of a region of interest in the proximal femur divided by region in the greater trochanter multiplied by 100. The region of interest in the proximal femur is specifically bordered with following anatomical landmarks: *linea intertrochanterica*, the superior-lateral line between *trochanter major* and metaphysis of the femur, horizontal line from the distal part of *trochanter minor* then connecting to the start of the first line.

Suh *et al.* (2012) found a correlation with this MCI and bone marrow mesenchymal cells from 32 patients who had a hip arthroplasty due to osteoarthritis. They aspirated three milliliter sample from the bone marrow of the proximal femur during the arthroplasty, then mononuclear cells were separated with Ficoll-Hypague and MSC quantity was calculated with Fibroblast Colony Forming Units (CFU-F) assays after one week of incubation in 37 degrees of Celsius. Mesenchymal stem cells tend to grow in fibroblast morphology colonies, thus the name CFU-F. According to Suh, each CFU-F represents a single MSC.

The results show that increased number of mononuclear cells significantly correlated with the increased number colony forming units, or in other words with Mesenchymal Stem Cells. Increased MNC quantity also correlated with the Marrow Conversion Index in inversely proportionally (when MNC increased, MCI decreased) manner. Marrow Conversion Index also had significant correlation with CFU-F in a same was as MNC. More studies of this method should be done.

7. Platelet-rich plasma as an alternative choice of treatment method

Platelet-rich plasma, known as PRP injections, are derived from the patient's own blood. PRP is a plasma with higher concentration of platelets than normal blood. This autologous peripheral blood sample is placed in a centrifuge, span for 4-15 minutes at 270-3200 rpm with exact time and rounds per minute depending on manufacturer instructions (Alves *et al.* 2018). The middle layer, so called

buffy coat layer containing platelets and white blood cells is collected. Additional substances can be added to the injections, such as calcium, thrombin or sodium bicarbonate, which all activate the platelets and cause them to release the growth factors from granules.

The exact mechanism how PRP works is not well known. The idea is that platelets contains growth factors, such as VEGF (vascular endothelial growth factor) and PDGF (platelet derived growth factor) which can cause increased angiogenesis and anabolism (Andia *et al.* 2013). According to Andia (2013), PRP has also anti-inflammatory effects by inhibiting nuclear factor kappa beta signaling pathways which are important inflammatory regulatory pathways. Other important effect is improvement of proliferation and activation of mesenchymal stem cells, thus increase the synthesis of matrix and collagen (Yu *et al.* 2019). The concentration of platelets in the PRP injection differs in different platelet separation systems. According to Karjalainen *et al.* (2017) PRP injections can have platelets two to five times more than in normal blood, and the final PRP injection has platelets 0.5 to 2 x 10^8 / ml. There is also different number of growth hormones and white blood cells in injections.

PRP therapy was developed in 1970s and 10 years later is was used in heart surgery to improve healing of the wound. After that the interest towards PRP has increased and now it is used in multiple different specialties, for example Sports Medicine, Plastic Surgery, Orthopedics, Dermatology, Maxillofacial Surgery, Gynecology, Urology, Ophthalmology etc. Especially interesting is the use of PRP therapy in the world of sport medicine. Highly known professional athletes, such as Tiger Woods (golf), Stephen Curry (NBA), Ray Lewis (NFL), Rafael Nada (Tennis) and many others have used PRP injections to fasten the recovery from different musculotendinous injuries. The World Anti-Doping Agency prohibits the use of growth factors, such as platelet derived growth factor and vascular endothelial growth factor, but the PRP injections are in a gray zone. According to a cross-sectional study of Kantrowitz *et al.* (2018) 93% of physicians of professional and collegiate teams state that they use PRP injections in their practices. Most of the players were NFL football players (60.4 players/season), less in National Hockey League (18 players/season). Team physicians listed hamstring injuries as the most common reason of PRP treatment.

Laudy *et al.* (2015) did a systematic review of the efficacy of PRP injections in osteoarthritis of the knee. Investigators included ten trials. In these trials, PRP injections were more effective method of pain reduction compared to placebo at the time period of six months post-injection. They

also found out that PRP injections were statistically more effective for pain reduction than hyaluronic acid at 6 months after injection. One must note that the follow-up time period was only six months. For better results a longer follow-up time period is needed.

Patel *et al.* (2013) did a double-blinded placebo-controlled study about the efficacy of the PRP injection to knee OA. There were three groups: first 50 people received only one PRP intra-articular injection, second group of 50 people received two PRP intra-articular injections in three-week interval and the third group of 50 people was the placebo group who received saline injection. Clinical outcome was evaluated in 6-month follow-up period by using WOMAC questionnaire and VAS scale. Both PRP injection groups gave statistically significant improvement in scores than the saline group, but there was no different between the groups of one and two injections.

Filardo *et al.* (2015) did a randomized control study with 12 months follow-up period of 192 patients with knee OA (KL grade 0-3) who received either three weekly intra-articular injections of PRP or Hyaluronic acid. Both injections gave beneficial effect. Different scoring systems were used to evaluate the outcome: IKDC, KOOS, VAS and Tegner score. All increased statistically significant amount. PRP had no adverse effects, only two HA patient had pain or swelling. Between the groups there were no different in any clinical scores, thus PRP injection is not superior to HA according to this study.

A fresh Scandinavian study about PRP versus HA injection for knee OA, KL grade 1-3. (Annanniemi *et al.* 2018) discusses how the PRP injections gives similar improvement in OA symptoms as HA gives. They investigated retrospectively 190 patients who received either PRP or HA injections, three injections approximately one week between injections (94 received PRP and 86 received HA). PRP injection contained four to eight times higher concentration of platelets than normal blood. Outcome was measured by using WOMAC and VAS scoring systems during 12-month follow-up period. The aim was to compare symptom's relief and time to arthroplasty in these two injections. They found that the arthroplasty rate was significantly higher (35% vs. 5.3%) in HA group than PRP group, odds ratio 4.4. Also, range of motion, VAS and WOMAC were worse in HA group than PRP group. PRP group had statistically delayed knee arthroplasty compared to HA group.

As a conclusion, there is still no consensus which one giver better outcome: PRP or Hyaluronic Acid. This is due to the investigation protocols, platelet concentrations and patient factors such as

age is different in different studies. One feature is commonly described by multiple studies: the effect of intra-articular PRP injection usually declines between the time period of 6 and 12 months.

Hede *et al.* (2019) did an interesting study of combining bone marrow aspirate and PRP on a collagen scaffold, which is a tissue-engineered material used for cartilage lesions. They did an arthroscopy with biopsies after one year to look how the tissue has regenerated. MRI was also done after 1, 2 and 3.5 years. Clinical scoring systems were also evaluated until two years post operatively. The final result was that the subjective scales, such as pain improved. The histology of the regenerated cartilage on the other hand showed fibrocartilage and fibrous tissue repairment instead of normal hyaline cartilage.

8. Bone marrow derived mononuclear cell therapy compared to HA in knee osteoarthritis

Gončars *et al.* (2018) did a study that insipred me to read more about mononuclear and mesenchymal stem cells. I believe in this method. I believe after a few decades when we have more understanding about these cells we will have a new card in our hand to fight against diseases.

In Gončars *et al.* (2018) research 56 patients with a knee osteoarthritis grade of K-L II to III and pain more than six months were included in the study. Exclusion criteria included age more than 75 years, different diseases such as diabetes, anemia, hepatic, lung or renal diseases, and use of immunosuppressants. So, basically the volunteers were relatively young and generally healthy from other major co-morbidities.

The volunteers were randomly divided into two groups: those who received bone marrow derived mononuclear cell injection and those who received three hyaluronic acid injections (one injection per week for three weeks). Average MNC yield was $45.56 \times 10^6 \pm 34.94 \times 10^6$ mononuclear cells MRI and x-ray was performed before injections and after 12 months follow-up period. Whole-organ Magnetic Resonance Imaging Score system was used to evaluate the MRI images. KOOS and KSS 0-100 scoring systems, including different objective parameters and clinical signs, were used to evaluate the functionality and pain.

There were no adverse effects. KOOS score improved totally by 15.3 points, pain score improved the most (20.5 points). Almost all KOOS subscales improved statistically at every follow-up point. KSS scores increased as well more than 20 points. X-ray showed no progression of OA after 12 months. MRI (WORMS score) improved statistically significant amount as well, a bit over one third of patients had improved cartilage changes and about half stayed in the same level of

degeneration. Compared to HA, the MNC group had significantly better scores almost in all KOOS subscales, most improvement was observed in pain. The research did not show correlation with the cell quantity and score improvement.

9. Other possible clinical use of MNC

MSC therapy is a hot topic in many specialties due to its wide range of possibilities and little amount of adverse effects. Arnold I. Caplan (2010) writes what are the possible future targets of the use of mesenchymal stem cells: due to mesenchymal stem cells are immunomodulatory by preventing the T-cells these cells could prevent the development of autoimmune diseases such as diabetes. They also have regenerative function, so they could enhance the differentiation of oligodendrocytes and myelin regeneration which are needed in the treatment of multiple sclerosis.

Saquillaro *et al.* (2016) summarizes the ongoing MSC trials in different diseases in their article. They say that according to the database of US National Institutes of Health total of 493 MSC-based clinical trials were found in June 2015. Nowadays the number is much more. The MSC-based clinical trials are presented in a Figure 6.

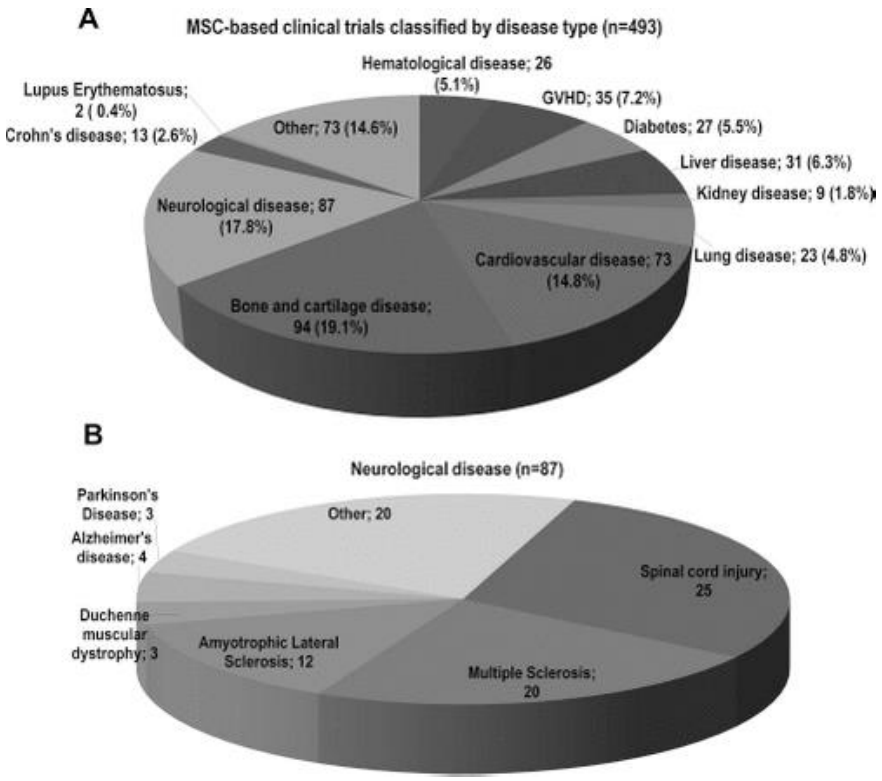

Figure 6 - Clinical trials of MSC classified according to A) disease, B) in different neurological diseases. Source: clinicaltrial.gov in Saquillaro et al (2016)

9.1. Increased function of myocardium after acute myocardial infarction

HEBE study, which is a large multicenter randomized trial of 200 patients who received intracoronary infusion of bone marrow mononuclear cells three to eight days after primary percutaneous coronary intervention (PCI) and stent placement or infusion of peripheral blood MNC after PCI and stent placement or just standard stent therapy without MNC infusion. In HEBE study, the global and regional left ventricle ejection fraction and volumes were investigated with MRI before randomization and four months after. After myocardial infarction the number of hematopoietic stem cells and endothelial progenitor cells increases, which may have an important function in myocardium repair, such as angiogenesis and others (Massa *et al.* 2005). The HEBE results did not show any different in LVEF and volume of the left ventricle between the groups. (Hirsch *et al.* 2011)

In REPAR-AMI trial intracoronary infusion of bone marrow MNC improved left ventricle ejection fraction (LVEF) after acute myocardial infarction compared to placebo in 204 patients. MNC improved LVEF 5.5 ± 7.3% and placebo increased LVEF 3.0 ± 6.5%. (Schächinger *et al.* 2006)

ASTAMI trial with 100 anterior wall acute myocardial infarction patient who received either bone marrow MNC four to eight days after AMI in addition to standard stent therapy or only standard stent therapy did not show significant difference in LVEF at six months measured by MRI or the size of infarct area measured by single photon emission computed tomography or the left ventricle end-diastolic volume (Lunde *et al.* 2008).

A meta-analysis by Delewi *et al.* (2012) about intracoronary bone marrow MNC therapy in ST-segment elevation myocardial infraction included 16 studies, total of 1641 patients. According to this meta-analysis the ejection fraction of left ventricle increased 2.55% compared to the control groups. People with ejection fraction less than 40% had the greatest benefit from MNC therapy. They found that the improvement in left ventricle was greater in patients less than 55 years of age. In addition, left ventricle end-diastolic volume was less in MNC patients.

9.2. Tendon repair

Already in 1998 a group of scientists, including Arnold I. Caplan, did an animal study with rabbit Achilles tendons about how MSC improves biomechanical properties when implanted as a part of tissue prosthesis into a gap of 1 cm in Achilles tendon. Compared to contralateral suture material the MSC containing Achilles tendon had twice better load-related structural properties and the

cross-sectional area was significantly thicker with better collagen alignment. All these show that MSC treated Achilles tendon heals faster and better than just suture material. (Young *et al.* 1998)

9.3. Healing of fistulas

Fistulas can be very problematic and cause significant problems. The New England Journal of Medicine reported a case where a bronchopleural fistula was healed after injection of mesenchymal stem cells derived from bone marrow (Petrella *et al.* 2015).

Crohn's disease can cause fistulae anywhere along the gastrointestinal tract, especially in perianal region. These fistulae can be very difficult to treat. Lee *et al.* (2013) investigated the use of adipose tissue derived stem cells in fistulae less than 2cm in size. The fistula was curetted and sutured with 2-0 vicryl and then cells were injected in the fistula with fibrin glue. 26 patients out of 33 had a successfully closed fistula after first injection. Similar results were observed by Garcia-Olmo *et al.* (2015).

Methods and Materials

Volunteers were originally included in Gončars *et al.* (2019) study "Treatment of Knee Osteoarthritis with Bone Marrow–Derived Mononuclear Cell Injection: 12-Month Follow-up" which was done in the Latvian State Hospital of Traumatology and Orthopedics and in the Cell Transplantation Center of the Pauls Stradiņš Clinical University hospital during 2013-2016. Some volunteers were also included originally in the study of Jakobsons *et al.* (2018) "Tissue evacuated during joint replacement as a source of mononuclear cells".

All patients voluntarily agreed to participate in the study and signed informed consent form. The informed consent was given according to Helsinki Declaration. This final thesis uses waived informed consent. Patient laboratory data which were taken during the original study are used retrospectively with the permission of the first and last author of the original study: Dr. Valdis Gončars and Dr. Andrejs Ērglis.

The total of 33 patients were included in the study. *DataMed* and *Ārsta Birojs* were used under the supervision and permission of Dr. Gončars. Patients' hemoglobin, erythrocytes, thrombocytes, age and sex parameters were collected from the laboratory data from patient database. Dr. Gončars kindly provided the data about bone marrow mononuclear cell quantity, $CD34^+$ quantity and the percentage of $CD34^+$ cells from his original study.

Cells in the original study were extracted from *crista iliaca* under local anesthesia or from excised tissue harvested during hip replacement operation. 45 mL of bone marrow was aspirated into heprain syringes and the material was shipped at room temperature to the laboratory where Good Manufacturing Practice standard requirements were used. NaCl 0.9% was used to dilute the aspirated material into 1:5. After filtration through 70 micrometer strainer the mononuclear cells were enriched with density gradient centrigugation 800 x g for 25 min with using Ficoll-Paque Premium. After separation fo the mononuclear cells they were washed three times with 45 mL of NaCl 0.9% with heparin 10 U/mL at the speed of 600 x g. The final material was resuspended in saline with heparin 10 000 U/L and it contained 5ml of mononuclear cells, no additional substances were added in the solution.

In the hip replacement group, 300 mL of liquid excised tissue was aspirated from AutoLog reservoir system, which included bone marrow, peripheral blood and adipose tissue. The aspirate was enriched with the same emthod as described before.

Results

33 patients were included in the study. Patients were originally selected by Gončars *et al.* (2019) according to their inclusion and exclusion criteria. It is worth mention that no adverse effects were found by Gončars *et al.*

IBM® SPSS® Statistics version 22.0, was used to calculate the results. A p-value ≤ 0.05 is considered as statistically significant.

Statistics

		Age	Erythrocytes	Hemoglobin	Thrombocytes	MNC	CD34	Percentage of CD34
N	Valid	33	33	33	33	33	33	33
	Missing	0	0	0	0	0	0	0
Mean		59,45	4,1315	124,91	224,36	41,4082	,762530	1,611210
Median		57,00	3,8900	123,00	224,00	27,9050	,540000	1,520000
Mode		57	5,10	101[a]	209[a]	27,91	,5400	1,5200
Skewness		-,360	,524	,077	,078	1,811	2,829	,898
Std. Error of Skewness		,409	,409	,409	,409	,409	,409	,409
Kurtosis		,102	-,582	-1,236	-,883	2,826	8,541	3,003
Std. Error of Kurtosis		,798	,798	,798	,798	,798	,798	,798
Minimum		34	3,06	91	125	8,54	,0000	,0000
Maximum		75	6,10	155	330	158,79	4,2000	4,7300

a. Multiple modes exist. The smallest value is shown

Sex

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	Female	19	57,6	57,6	57,6
	Male	14	42,4	42,4	100,0
	Total	33	100,0	100,0	

Figure 7 – Epidemiological data

Epidemiological statistic results show that from 33 patients 19 were female (57.6 %). The mean (average) age was 59.45 years (Standard Deviation (SD) ± 9.421 years). The range of the age was from 34 years to 75 years. The mean erythrocyte level was 4.13 x 10^{12} / L (SD ± 0.795 x 10^{12} / L)

with minimum value 3.06 x 10^{12} / L and maximum value 6.10 x 10^{12} / L. The mean hemoglobin level was 124.91 g/L (SD ± 19.06 g/L) with minimum level 91 g/L and maximum level 155 g/L. Mean thrombocyte level was 224.36 x 10^{9} / L (SD ± 61.66 x 10^{9} / L) with minimum level 125 x 10^{9} / L and maximum level 330 x 10^{9} / L.

Mononuclear cells had mean value of 41.408 x 10^{6} (SD ± 36.471 x 10^{6}) with a yield of 8.54 x 10^{6} - 158.79 x 10^{6}. $CD34^{+}$ cells had a mean value of 0.7625 x 10^{6} (SD ± 0.9225 x 10^{6}). The mean percentage of $CD34^{+}$ was 1.611 % (SD ± 0.9319 %).

Descriptive statistics of nominal data (age) is presented as a pie chart (figure 7). Descriptive statistics of continuous data (age, erythrocytes, hemoglobin, thrombocytes, MNC, $CD34^{+}$, percentage of $CD34^{+}$ cells) are presented with a histogram with normal curve (figures 8 to 13).

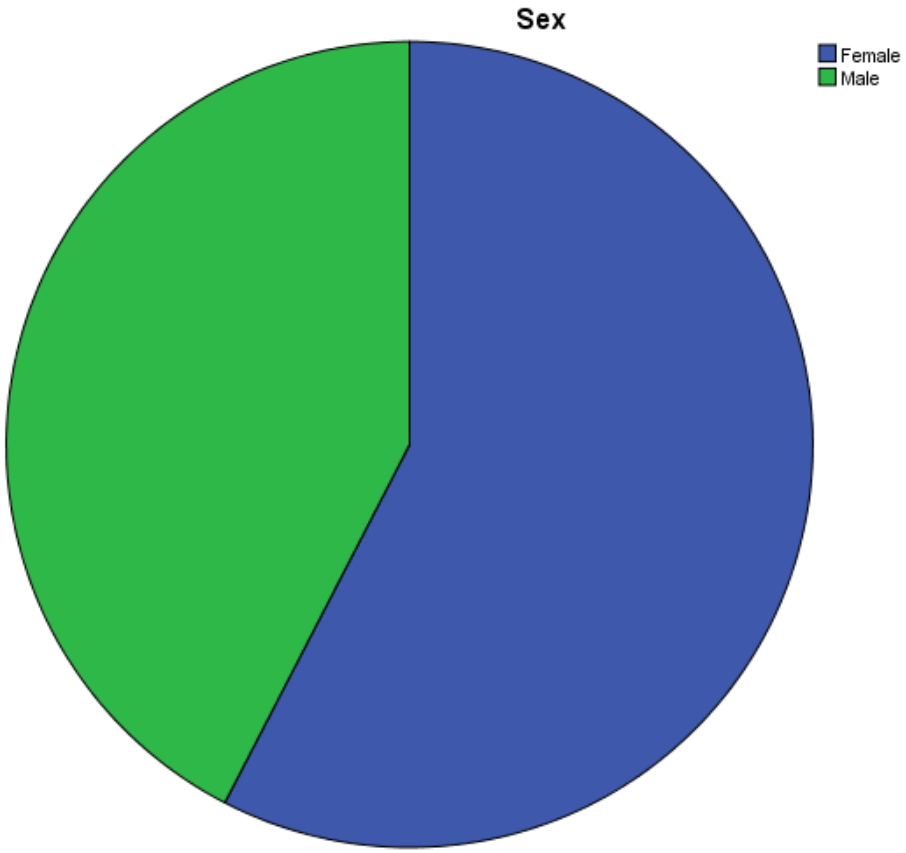

Figure 8 - Distribution of the sex

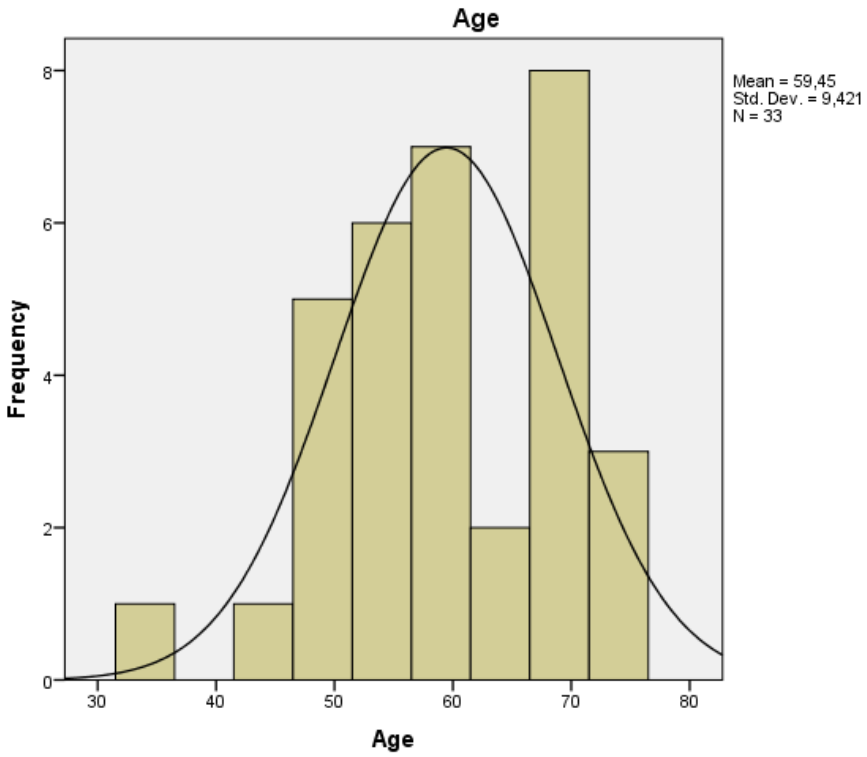

Figure 9- Age distribution

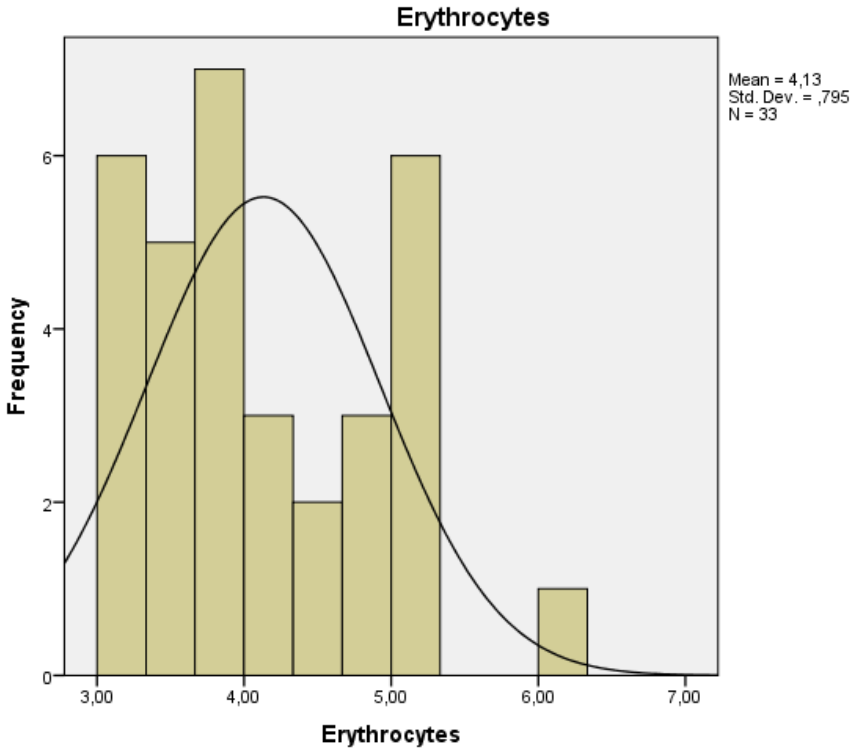

Figure 10 - Erythrocyte distribution

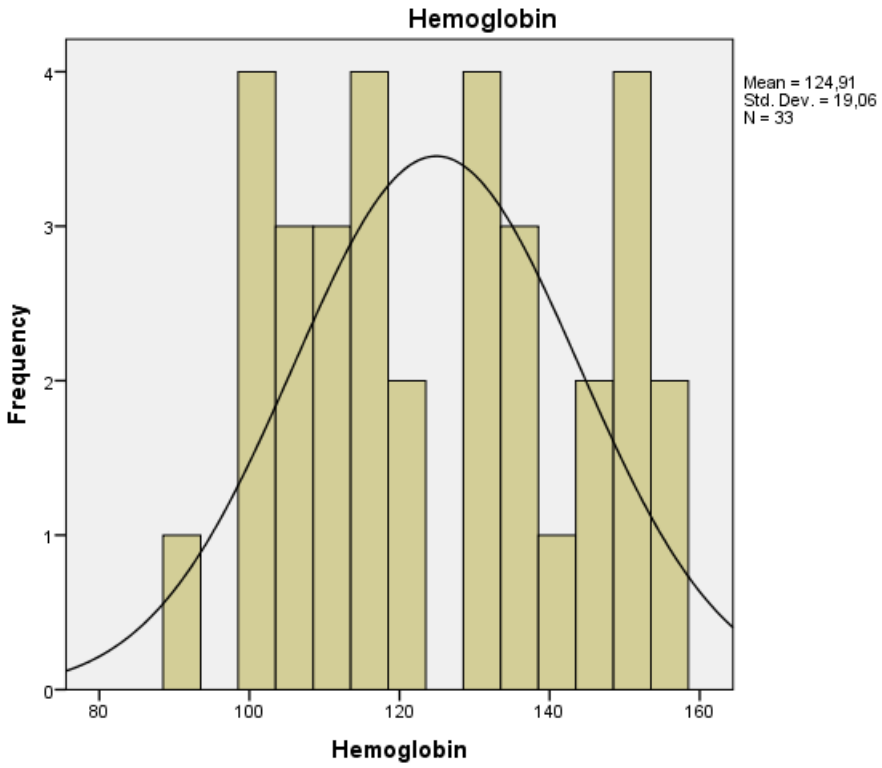

Figure 11 - Hemoglobin distribution

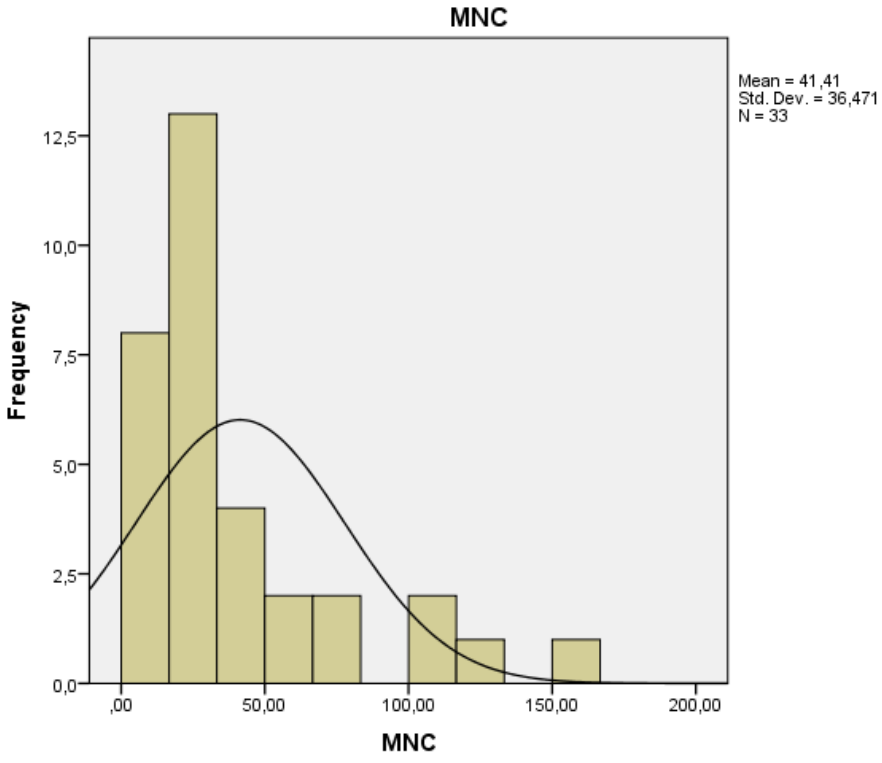

Figure 12 - Mononuclear cell distribution

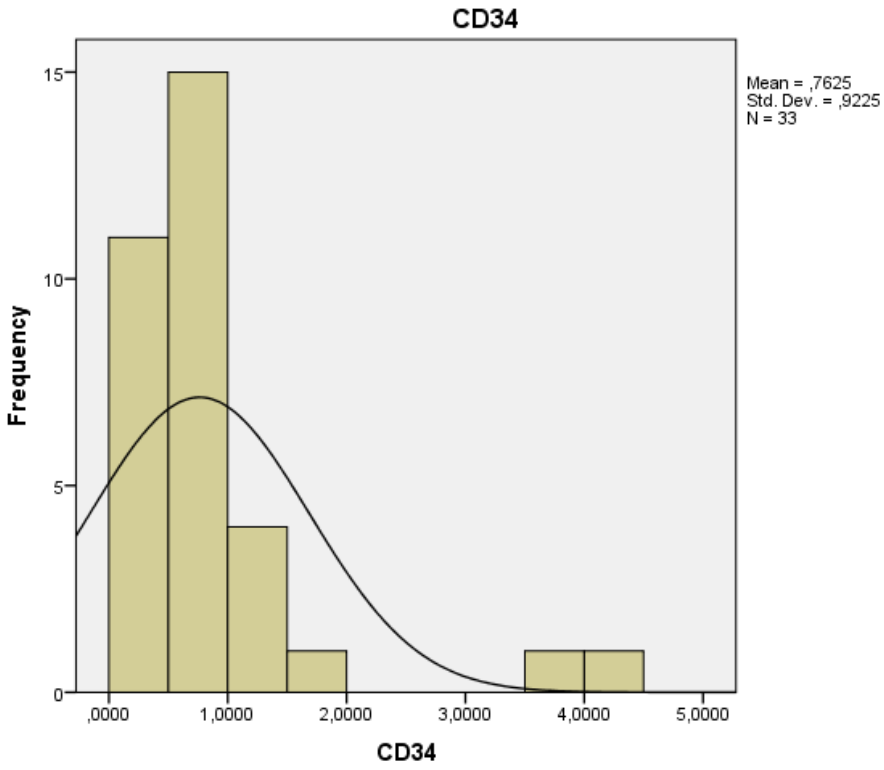

Figure 13 - CD34⁺distribution

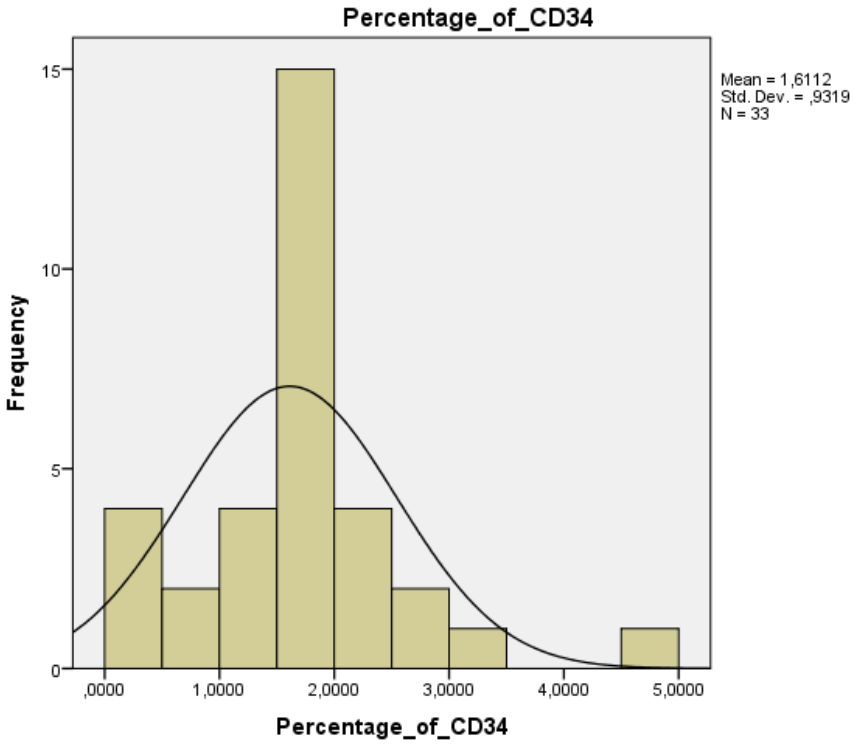

Figure 14 - distribution of the percentage of CD34⁺ cells

Univariate analysis of two continuous non-normally distributed data was calculated by using Spearman correlation coefficient.

Correlations

			Age	MNC
Spearman's rho	Age	Correlation Coefficient	1,000	,187
		Sig. (2-tailed)	.	,297
		N	33	33
	MNC	Correlation Coefficient	,187	1,000
		Sig. (2-tailed)	,297	.
		N	33	33

Spearman correlation coefficient showed weak positive but statistically non-significant correlation between age and MNC (r_s = 0,187, p = 0,297). H_0 is accepted.

Correlations

			MNC	Erythrocytes
Spearman's rho	MNC	Correlation Coefficient	1,000	-,158
		Sig. (2-tailed)	.	,379
		N	33	33
	Erythrocytes	Correlation Coefficient	-,158	1,000
		Sig. (2-tailed)	,379	.
		N	33	33

Spearman correlation coefficient showed weak negative but statistically non-significant correlation between erythrocytes and MNC (r_s = -0,158, p = 0,379). H_0 is accepted.

Correlations

			MNC	Hemoglobin
Spearman's rho	MNC	Correlation Coefficient	1,000	-,181
		Sig. (2-tailed)	.	,314
		N	33	33
	Hemoglobin	Correlation Coefficient	-,181	1,000
		Sig. (2-tailed)	,314	.
		N	33	33

Spearman correlation coefficient showed weak negative but statistically non-significant correlation between hemoglobin level and MNC (r_s = -0,181, p = 0,314). H_0 is accepted.

Correlations

			MNC	Thrombocytes
Spearman's rho	MNC	Correlation Coefficient	1,000	,132
		Sig. (2-tailed)	.	,464
		N	33	33
	Thrombocytes	Correlation Coefficient	,132	1,000
		Sig. (2-tailed)	,464	.
		N	33	33

Spearman correlation coefficient showed weak positive but statistically non-significant correlation between thrombocytes and MNC (r_s = 0,132, p = 0,464). H_0 is accepted.

The correlation with the quantity of mononuclear cells (MNC) according to Spearman		
	Spearman correlation coefficient (r_s)	p-value
Age	0.187	0.297
Erythrocytes	- 0.158	0.379
Hemoglobin	- 0.181	0.314
Thrombocytes	0.132	0.464

Figure 15 - Correlation of factors with MNCs

Correlations

			CD34	Age
Spearman's rho	CD34	Correlation Coefficient	1,000	,102
		Sig. (2-tailed)	.	,572
		N	33	33
	Age	Correlation Coefficient	,102	1,000
		Sig. (2-tailed)	,572	.
		N	33	33

Spearman correlation coefficient showed weak positive but statistically non-significant correlation between age and $CD34^+$ cells (r_s = 0,102, p = 0,572). H_0 is accepted.

Correlations

			CD34	Erythrocytes
Spearman's rho	CD34	Correlation Coefficient	1,000	-,147
		Sig. (2-tailed)	.	,416
		N	33	33
	Erythrocytes	Correlation Coefficient	-,147	1,000
		Sig. (2-tailed)	,416	.
		N	33	33

Spearman correlation coefficient showed weak negative but statistically non-significant correlation between erythrocytes and $CD34^+$ cells (r_s = -0,147, p = 0,416). H_0 is accepted.

Correlations

			CD34	Hemoglobin
Spearman's rho	CD34	Correlation Coefficient	1,000	-,209
		Sig. (2-tailed)	.	,242
		N	33	33
	Hemoglobin	Correlation Coefficient	-,209	1,000
		Sig. (2-tailed)	,242	.
		N	33	33

Spearman correlation coefficient showed weak negative but statistically non-significant correlation between hemoglobin and $CD34^+$ cells (r_s = -0,209, p = 0,242). H_0 is accepted.

Correlations

			CD34	Thrombocytes
Spearman's rho	CD34	Correlation Coefficient	1,000	,048
		Sig. (2-tailed)	.	,790
		N	33	33
	Thrombocytes	Correlation Coefficient	,048	1,000
		Sig. (2-tailed)	,790	.
		N	33	33

Spearman correlation coefficient showed weak positive but statistically non-significant correlation between thrombocytes and $CD34^+$ cells (r_s = 0,048, p = 0,790). H_0 is accepted.

The correlation with the quantity of $CD34^+$ cells according to Spearman		
	Spearman correlation coefficient (r_s)	p-value
Age	0.102	0.572
Erythrocytes	- 0.147	0.416
Hemoglobin	- 0.209	0.242
Thrombocytes	0.048	0.790

Figure 16 - Correlation of factors with CD34⁺cells

Correlations

			Percentage_of_CD34	Age
Spearman's rho	Percentage_of_CD34	Correlation Coefficient	1,000	-,075
		Sig. (2-tailed)	.	,677
		N	33	33
	Age	Correlation Coefficient	-,075	1,000
		Sig. (2-tailed)	,677	.
		N	33	33

Spearman correlation coefficient showed weak negative but statistically non-significant correlation between age and percentage of $CD34^+$ cells (r_s = -0.75, p = 0,677). H_0 is accepted.

Correlations

			Percentage_of_CD34	Erythrocytes
Spearman's rho	Percentage_of_CD34	Correlation Coefficient	1,000	,213
		Sig. (2-tailed)	.	,235
		N	33	33
	Erythrocytes	Correlation Coefficient	,213	1,000
		Sig. (2-tailed)	,235	.
		N	33	33

Spearman correlation coefficient showed weak positive but statistically non-significant correlation between erythrocytes and percentage of $CD34^+$ cells (r_s = 0.213, p = 0,235). H_0 is accepted.

Correlations

			Percentage_of_CD34	Hemoglobin
Spearman's rho	Percentage_of_CD34	Correlation Coefficient	1,000	,093
		Sig. (2-tailed)	.	,607
		N	33	33
	Hemoglobin	Correlation Coefficient	,093	1,000
		Sig. (2-tailed)	,607	.
		N	33	33

Spearman correlation coefficient showed weak positive but statistically non-significant correlation between hemoglobin and percentage of $CD34^+$ cells (r_s = 0,093, p = 0,607). H_0 is accepted.

Correlations

			Percentage_of_CD34	Thrombocytes
Spearman's rho	Percentage_of_CD34	Correlation Coefficient	1,000	,005
		Sig. (2-tailed)	.	,979
		N	33	33
	Thrombocytes	Correlation Coefficient	,005	1,000
		Sig. (2-tailed)	,979	.
		N	33	33

Spearman correlation coefficient showed weak positive but statistically non-significant correlation between thrombocytes and percentage of $CD34^+$ cells (r_s = 0,005, p = 0,979). H_0 is accepted.

The correlation with the percentage of $CD34^+$ cells according to Spearman		
	Spearman correlation coefficient (r_s)	p-value
Age	- 0,075	0,677
Erythrocytes	0,213	0,235
Hemoglobin	0,093	0,607
Thrombocytes	0,005	0,979

Figure 17 - Correlation of factors with the percentage of $CD34^+$ cells

The association between a nominal variable (sex) and continuous variable (MNC and the percentage of $CD34^+$ cells) was calculated with independent sample T-test.

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
MNC	Equal variances assumed	,087	,770	,467	31	,644	6,07827	13,00556	-20,44675	32,60329
	Equal variances not assumed			,474	29,455	,639	6,07827	12,82863	-20,14164	32,29818

Independent sample T-test between MNC and sex shows non-significant "Equal variances assumed" meaning the significance p-value is 0,639 which is statistically non-significant. We accept H_0.

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Percentage Of CD34	Equal variances assumed	,754	,392	-,771	31	,447	-,2546169	,3303327	-,9283350	,4191012
	Equal variances not assumed			-,730	21,946	,473	-,2546169	,3487439	-,9779707	,4687368

Independent sample T-test between percentage of $CD34^+$ cells and sex shows non-significant "Equal variances assumed" meaning the significance p-value is 0,473 which is statistically non-significant. We accept H_0.

A table of the results of univariate analysis.

The correlation with the quantity of mononuclear cells (MNC) according to Spearman		
	Spearman correlation coefficient (r_s)	p-value
Age	0.187	0.297
Erythrocytes	- 0.158	0.379
Hemoglobin	- 0.181	0.314
Thrombocytes	0.132	0.464
The correlation with the quantity of $CD34^+$ cells according to Spearman		
	Spearman correlation coefficient (r_s)	p-value
Age	0.102	0.572
Erythrocytes	- 0.147	0.416
Hemoglobin	- 0.209	0.242
Thrombocytes	0.048	0.790
The correlation with the percentage of $CD34^+$ cells according to Spearman		
	Spearman correlation coefficient (r_s)	p-value
Age	- 0,075	0,677
Erythrocytes	0,213	0,235
Hemoglobin	0,093	0,607
Thrombocytes	0,005	0,979
The association between a nominal variable (sex) and continuous variable calculated with independent sample T-test.		
	p-value	
MNC	0,639	
Percentage of $CD34^+$ cells	0,473	

Figure 18 – A table of the results of univariate analysis

Multivariate analysis was not performed due to none of the univariate analysis were significantly ($p \leq 0.1$) related to the dependent variables.

Scatter plot graph of the results

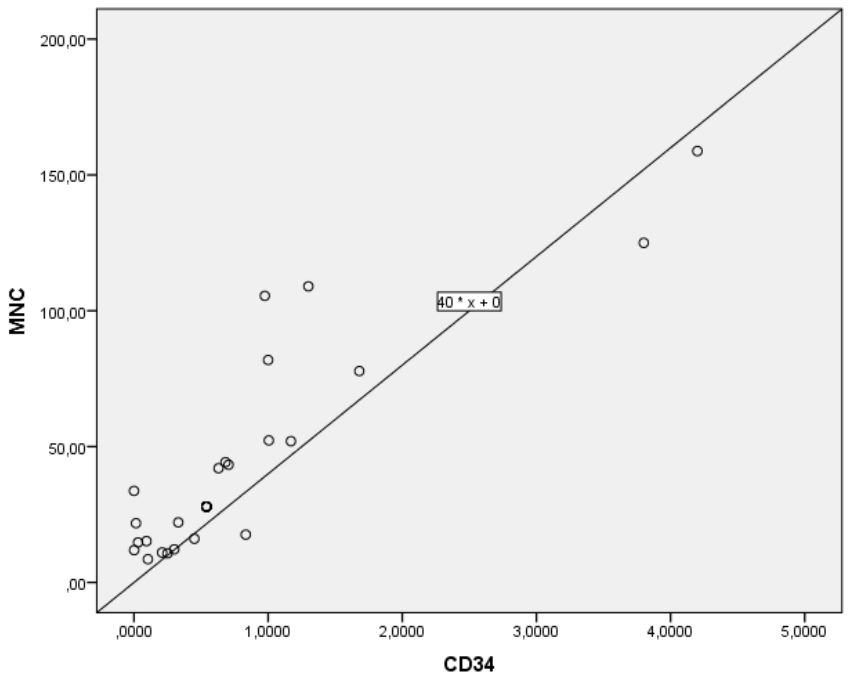

Figure 19 - The scatter plot graph of mononuclear cells and CD34+ cells

The scatter plot graph of mononuclear cells and $CD34^+$ cells shows a significant correlation between these two continuous variables. Scatter plot graphs of MNC, $CD34^+$ cells and the percentage of $CD34^+$ cells versus continuous variables are shown below.

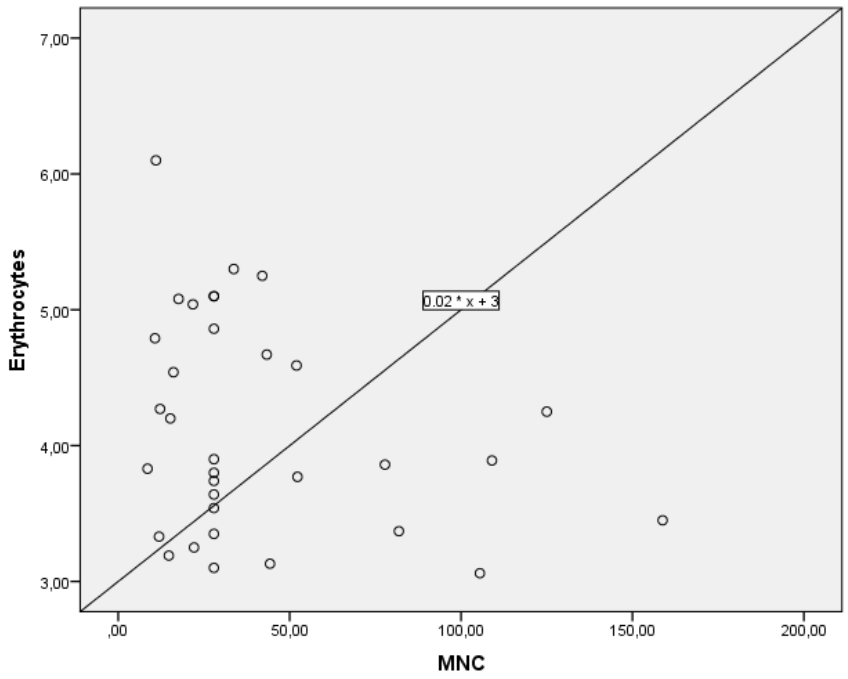

Figure 20 - The scatter plot graph of mononuclear cells and erythrocytes.

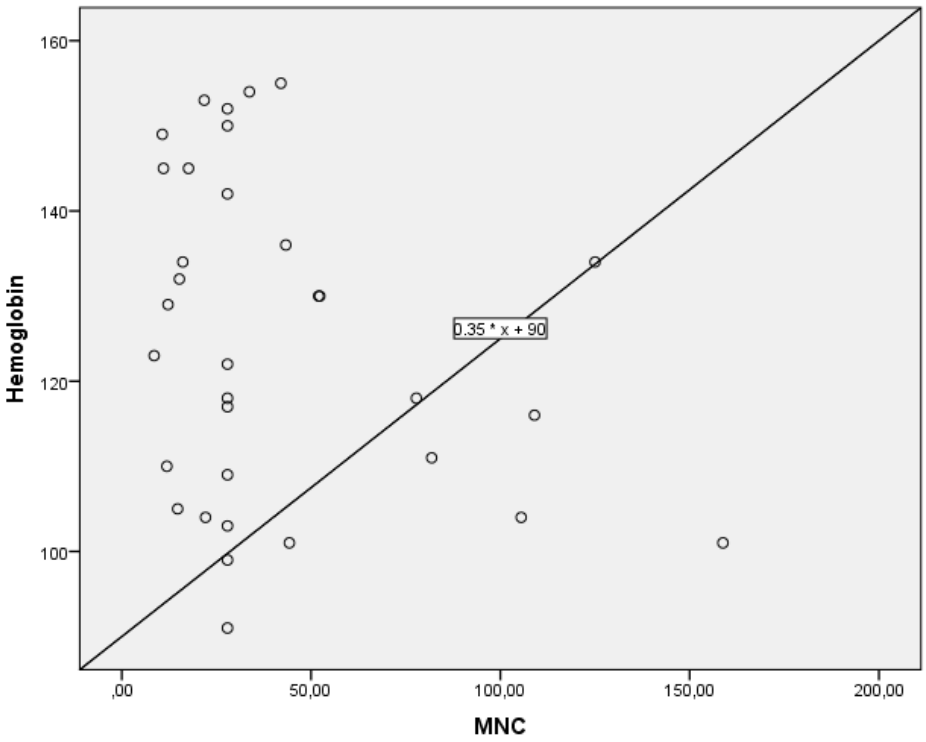

Figure 21 - The scatter plot graph of mononuclear cells and hemoglobin.

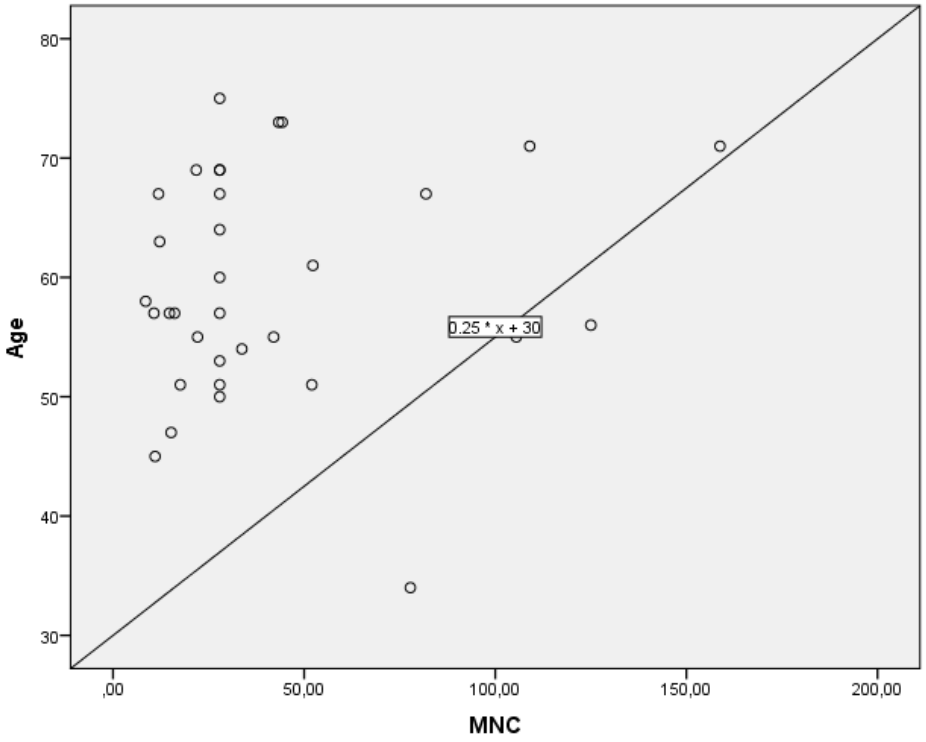

Figure 22 - The scatter plot graph of mononuclear cells and age.

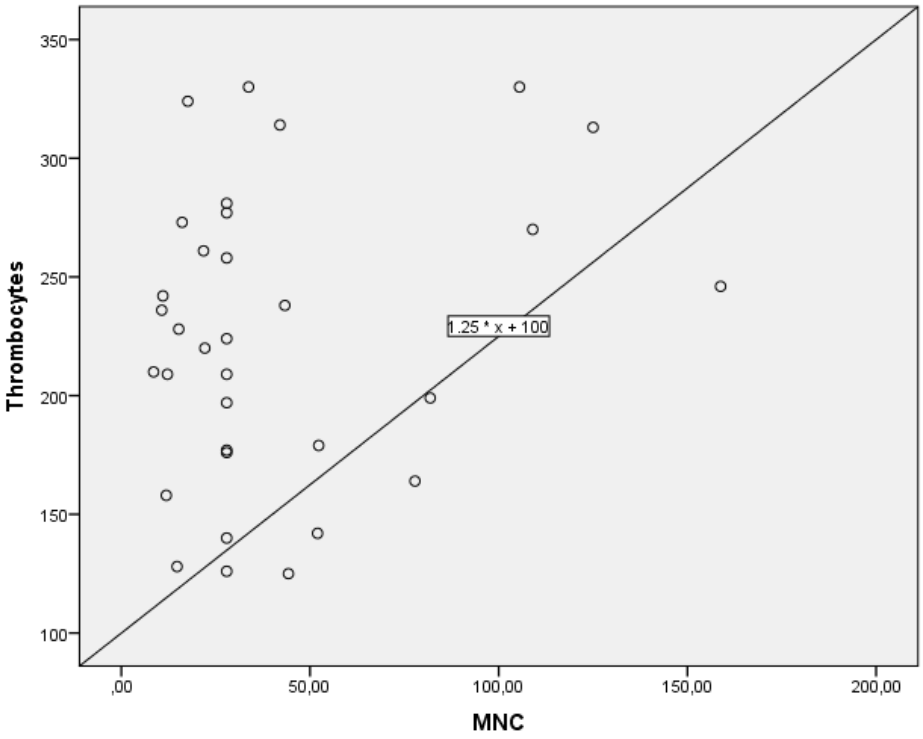

Figure 23 - The scatter plot graph of mononuclear cells and thrombocytes.

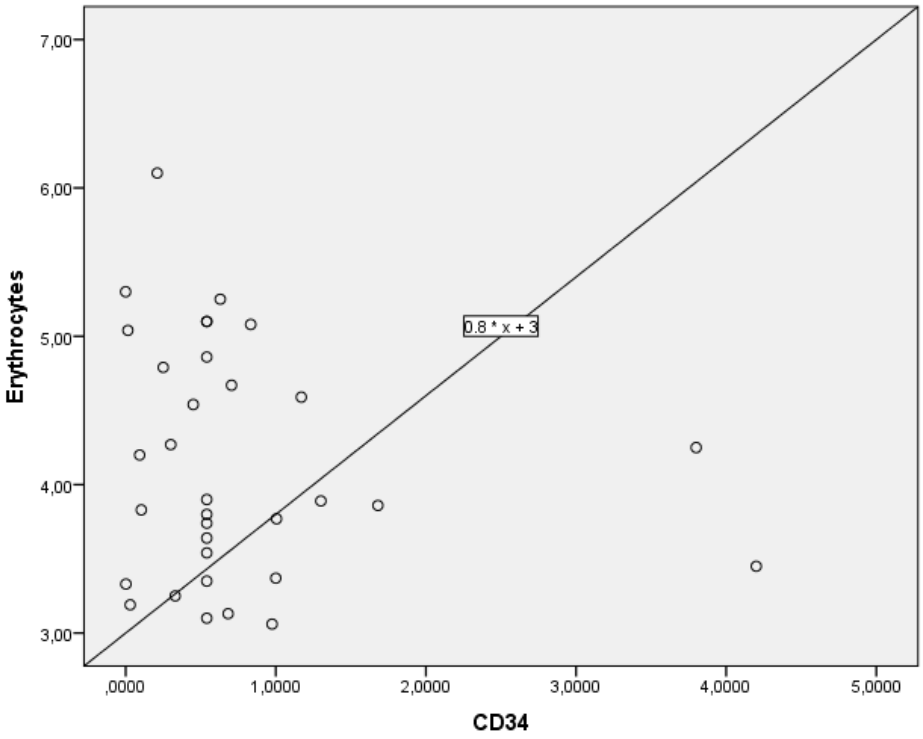

Figure 24 - The scatter plot graph of CD34⁺ cells and erythrocytes.

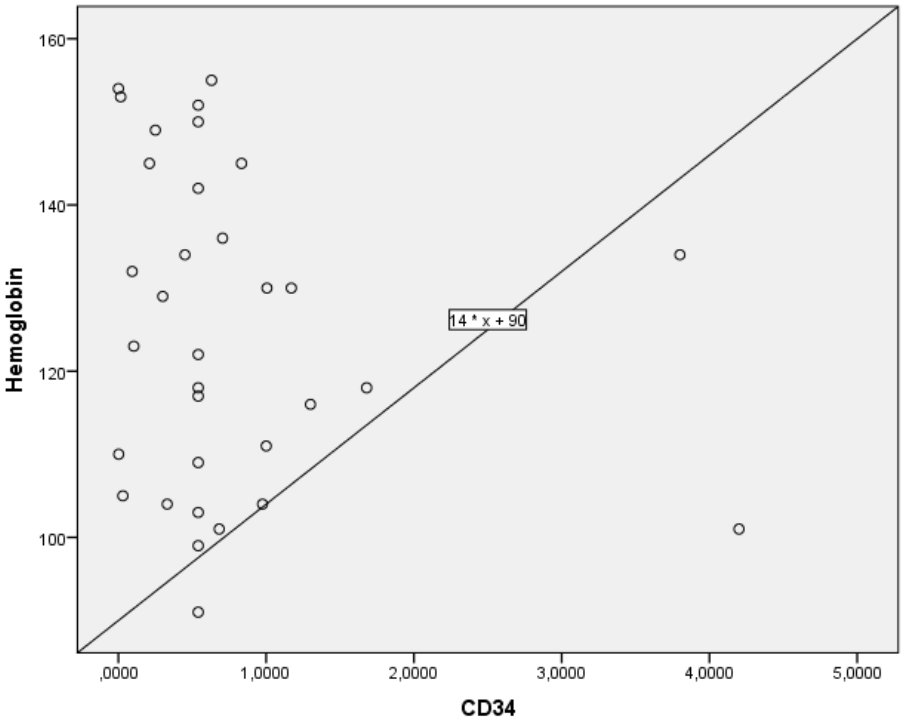

Figure 25 - The scatter plot graph of CD34+ cells and hemoglobin.

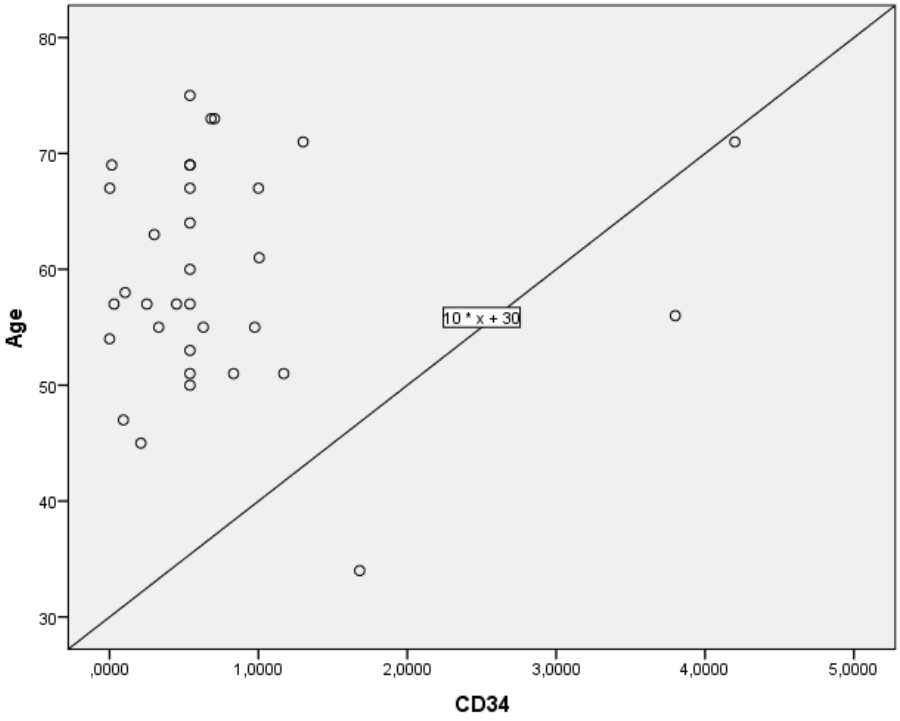

Figure 26 - The scatter plot graph of CD34+ cells and age.

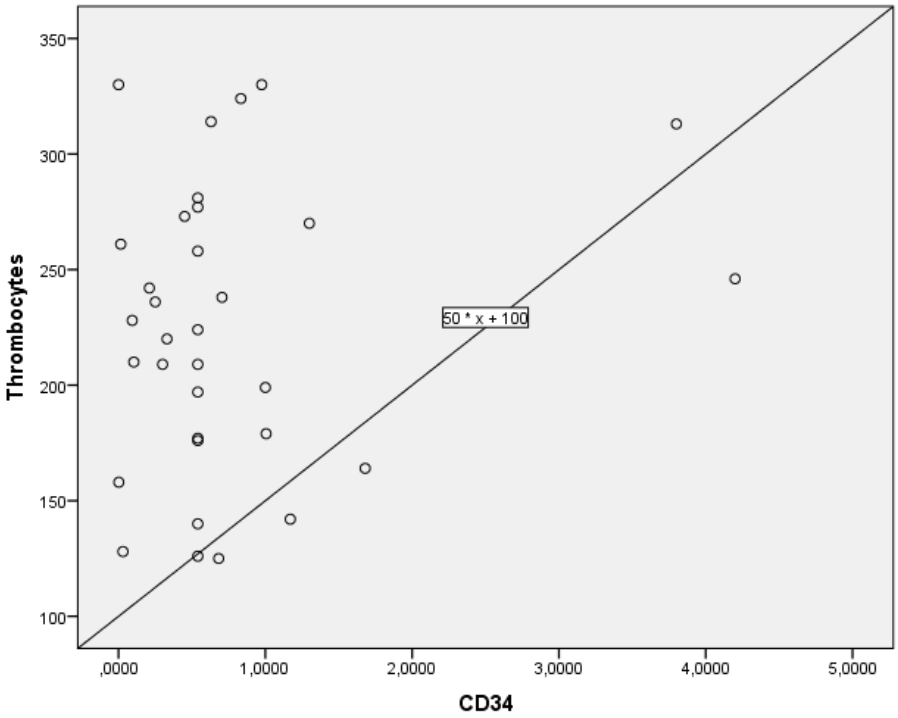

Figure 27 - The scatter plot graph of CD34+ cells and thrombocytes.

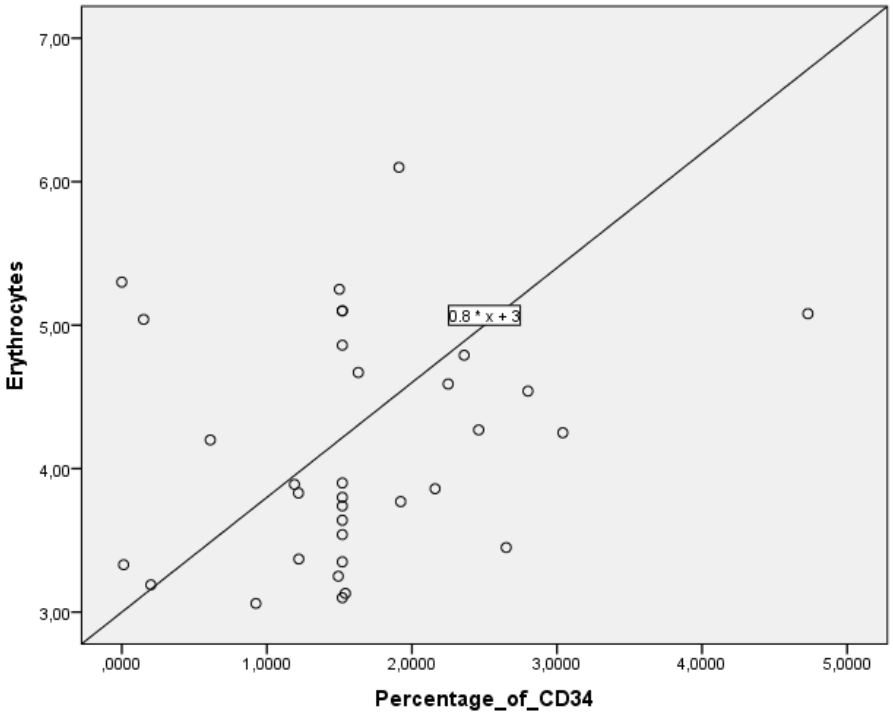

Figure 28 - The scatter plot graph of percentage of CD34+ cells and erythrocytes.

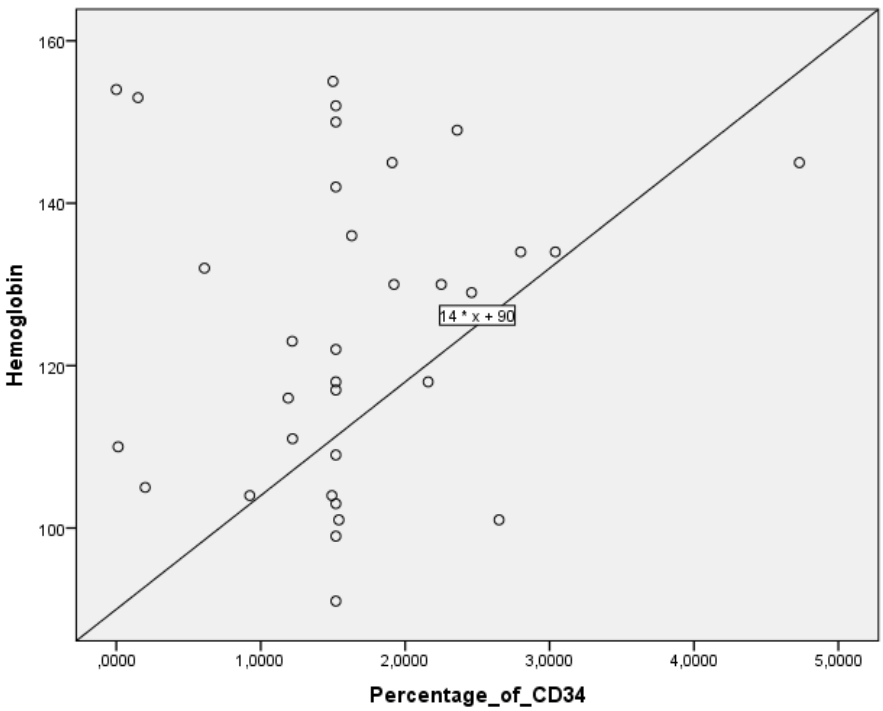

Figure 29 - The scatter plot graph of percentage of CD34+ cells and hemoglobin.

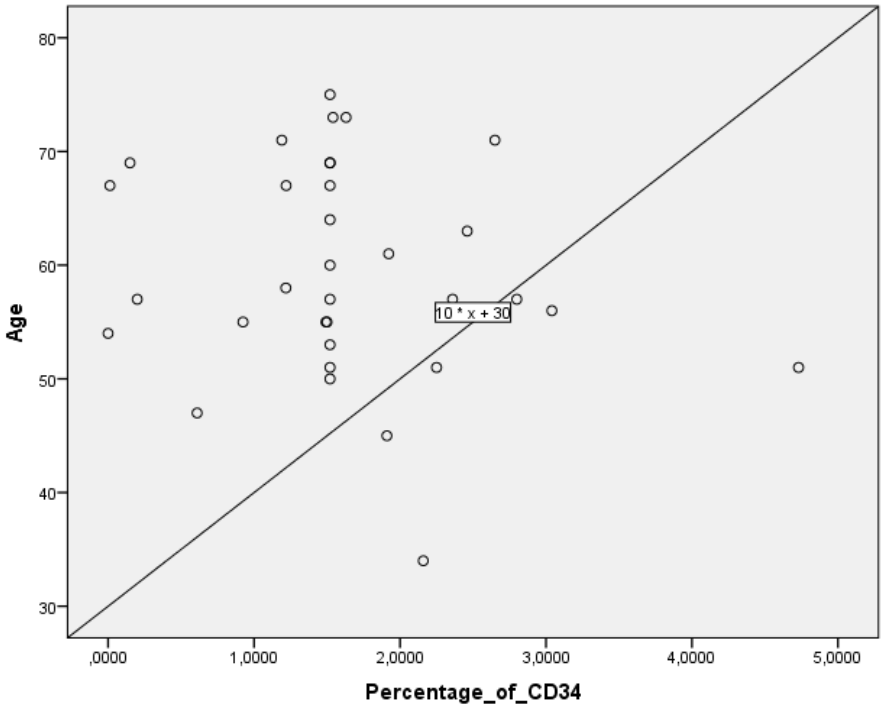

Figure 30 - The scatter plot graph of percentage of CD34+ cells and age.

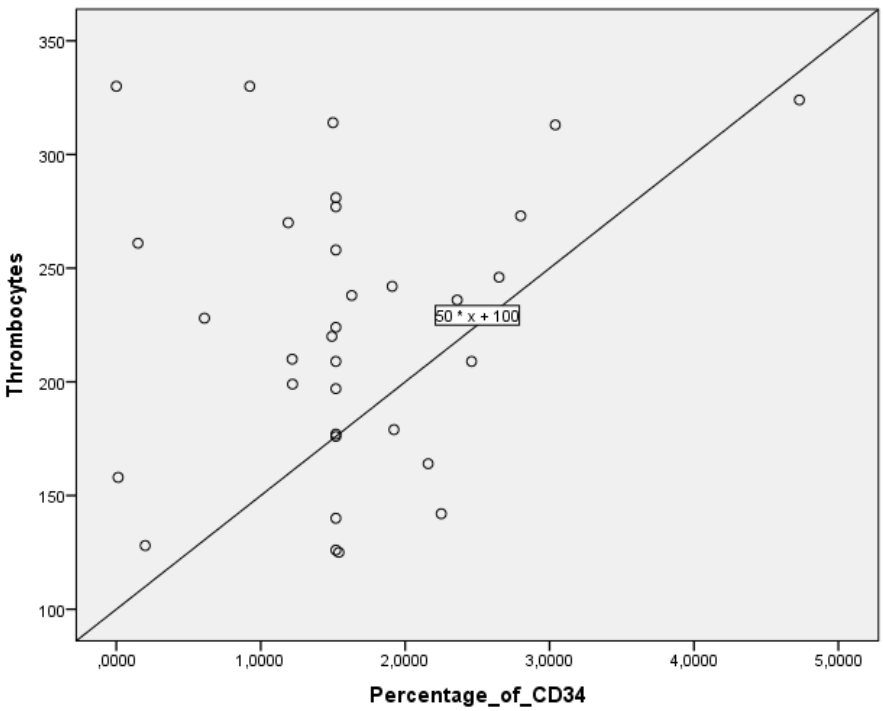

Figure 31 - The scatter plot graph of percentage of CD34+ cells and thrombocytes.

Results show that none of the investigated factors, including age, sex, erythrocyte level, hemoglobin level and thrombocyte level have no correlation with the quantity of mononuclear cells, $CD34^+$ cells or the percentage of $CD34^+$ cells.

Discussion

There is still a lack of knowledge of what is the optimal quantity of cells used and what factors affects the quality and quantity of these cells. These are examples of hot topics in the field of mononuclear cell therapy.

The goal of my study is to investigate patient's factors that could influence the quantity or quality of mononuclear cells and $CD34^+$ cells which were harvested from the bone marrow of a selected group of people or from the excised tissue harvested during joint replacement. The goal of the study was also the evaluation of the used laboratory method and its potential effect to the quantity and quality of the mononuclear cells. The hypothesis of my study is that patient factors, such as age, sex, erythrocytes, thrombocytes and hemoglobin correlate with the number of mononuclear cells and $CD34^+$ cells extracted from the bone marrow.

Some factors are proven to influence the quality and quantity of MNC. These are contamination with erythrocytes (Assums *et al.* 2010), apoptotic cell content (Monquet et al 2011), different steps in washing and centrifugation speed (van Beem RT *et al.* 2008). Dimmeler *et al.* (2008) states that patients with type I and type II diabetes mellitus (DM) have a smaller number of hematopoietic stem cells, defined as the $CD34^+$ cells and endothelial progenitor cells. Loomans *et al.* (2004) found 44% decrease of EPC in type 1 DM patient compared to non-diabetic control group. In addition, they found that increased HbA_{1C} causes decreased number of EPC.

Adipose tissue has greater number of MSC per tissue weight than bone marrow aspiration according to Kiviranta *et al.* (2012). This makes the adipose tissue an interesting target of investigation, especially because adipose tissue is easily accessible.

Ficoll-Paque density gradient centrifugation medium is one of the most commonly used density gradient medium in studies to enrich the mononuclear cell population (Pösel *et al.* 2012). Material e.g. bone marrow aspirate is carefully layered on the Ficoll-Paque solution which has been adjusted to the temperature of 18 to 20 degrees of Celsius. Then the solution is centrifugated with a speed of 600 to 800 g, which gives significantly better cell recovery compared to speed of 250 g according to Van Beem RT *et al.* (2008). At high temperature, around 37 degrees of Celsius, aggregation of erythrocytes and mononuclear cells are increased. At low temperature, around 4 degrees of Celsius, the aggregation is decreased but separation time increases which causes worse mononuclear cell separation.

Jaatinen (2007) recommends a centrifugation speed of 400 g for 40 minutes without brake when separating mononuclear cells and when washing the mononuclear cells twice in 40ml of phosphate-buffered saline with a speed of 300 g for 10 minutes with break. GE Healthcare Bio-Sciences AB (2014) recommends also centrifugation at 400 g for 30 to 40 minutes at room temperature without brake.

Jaatinen (2007) describes the problem of erythroid cells. These cells do not sediment in the bottom layer as expected. Some nucleated erythroid progenitor cells may stay at the layer of mononuclear cells causing harm. Jaatinen also consider that erythrocytes may aggregate and adhere to lymphocytes causing sedimentation of lymphocytes in the bottom. This can be reduced by diluting the blood sample. Jaatinen recommends diluting the blood sample 1:4 by using a phosphate-buffered saline to reduce aggregation of erythrocytes. Assums *et al.* (2010) left ventricle ejection fraction four months after intracoronary bone marrow mononuclear cell therapy was significantly reduced and reduction correlated with the contamination of the cell product by erythrocytes. Jaatinen writes that the blood volume and tube diameter are critical for successful isolation of pure mononuclear cells, because increasing the height of the blood sample increases the contamination with erythrocytes. GE Healthcare Bio-Sciences AB (2014) keeps 2.4cm of Ficoll-Paque media and 3.0cm of blood sample as a standard.

Injected bone marrow mononuclear cells may poorly survive locally. These apoptotic cells could release microparticles to induce the apoptosis of other cells nearby, thus impair the efficacy of the therapy (Monquet *et al.* 2011). Function can also be affected by changes in temperature of the storage, buffer solution choice or use of plasma from the patients during the cell processing and isolation These impairs the CFU capacity, thus the functionality, capacity to migrate toward chemoattractant and angiogenesis in hindlimb ischemia patients (Assums *et al.* 2007).

Yong Sang Kim *et al.* (2015) found that clinical failure of MSC therapy was significantly influenced by age over 60 years and the cartilage lesion over 6.0 cm^2 significantly influenced the clinical outcome if compared to lesion size less than 6.0 cm^2.

Nobody knows what the optimal quantity of mononuclear cells and mesenchymal stem cells is to achieve the best clinical outcome. According to the literature and recommendation of the cell processing, Gončars et al used perfect centrifugation speed (800 g) and time (25 min). They also diluted the sample into 1:5 to reduce the erythrocyte contamination as recommended. They shipped

the bone marrow aspirate in room temperature, which is also ideal. Their exclusion criteria was diabetes, so they eliminated that patient factor as well.

Limitations of the study is the use of two different protocols of cell harvesting: bone marrow aspirate and excised material during joint replacement. Another limitation is the number of patients.

In the future, it would be great to have a study where only one cell harvesting method is used. The height of the blood sample should be clearly defined. The time period from the bone marrow aspirate to cell processing with Ficoll-Paque medium should also be as minimum as possible to avoid the clamping of mononuclear cells and mesenchymal stem cells with erythrocytes. Also, bone marrow density measurement with MRI could be done beforehand to evaluate the correlation with cell mononuclear cell quantity. Marrow Conversion Index calculated from proximal femur MRI had a correlation with the quantity of Mononuclear Cells and Mesenchymal Stem Cells in Suh *et al.* (2012) study.

Conclusions

Hemoglobin level, erythrocyte level, thrombocyte level, age and sex do not correlate with the quantity of mononuclear cells, $CD34^+$ cells or the percentage of $CD34^+$ cells.

The cell processing method, used in Gončars and Jakobsons studies, was according to the best current knowledge.

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Ethics Committee Evaluation

All patients voluntarily agreed to participate in the study and signed informed consent form. The informed consent was given according to Helsinki Declaration. This final thesis uses waived informed consent. Patient laboratory data which were taken during the original study are used with the permission of the first and last author of the original study: Dr. Valdis Gončars and Prof. Andrejs Ērglis.

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