UNIVERSITY OF LIVERPOOL

THE DIFFERENCE IN SYNOVITIS (SYNOVIAL MEMBRANE) AND ANGIOGENESIS BETWEEN YOUNG OBESE, YOUNG LEAN AND OLD LEAN PEOPLE WITH PRIMARY KNEE OSTEOARTHRITIS

THESIS SUBMITTED IN ACCORDANCE WITH THE REQUIREMENT OF THE

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ABSTRACT

Background: OA is a complex disorder with multiple risk factors including obesity. Obesity has been implicated for early OA due to mechanical and metabolic effect of the fat through low-grade inflammation. Also, synovitis and inflammation have gained a lot of popularity in the research field as a cause of OA.

Aims: 1: To determine the effect of BMI on the synovial vascularity and inflammation among different age groups with advanced OA. 2- To determine the effect of fat cell size on the vascularity and inflammation. 3- To assess the difference in synovitis among young obese, young lean and old lean people with advanced OA.

Methodology: A total of 17 patients (matched for gender, the advanced stage of OA requiring total knee arthroplasty, KL score, comorbidities, ASA grade, medications and functional outcome score) have been recruited into three almost equal groups based on age (young <60, old >70) and BMI (lean <30, obese >30). Group 1 (young obese (n=6)) with age and BMI mean (SD) 53.3 (2.33) and 38.53 (1.16) respectively. Group 2 (young lean (n=5)) with age and BMI 55.60(4.5) and 27.01(1.59) respectively. Group 3 (old lean (n=6)) with age and BMI 75.33 (3.2) and 25.64 (2.5) respectively. The synovial tissues obtained during surgery were examined using H&E staining and IHC staining of vWF, CD34, CD68, CD3, CD20 and vimentin. Quantitative assessment of each marker was done using image J software in the synovial tissue sections, fat tissue sections and combined total sections. Also fat cell size was measured and divided into small and big fat cells. The IHC markers were also analyzed based on the fat cell size. Other manual counts were done for the vessels in the synovial and fat sections. Vessels diameter and distance to the synovial surface were measured too.

Results: The overall vascularity did not show any significant difference across the groups (p=0.468). There was significant difference of vimentin across the groups (p=0.006). The other inflammatory markers showed a trend of increasing with increase BMI. However, macrophages, T cells, B cells and fibroblasts were significantly higher in the presence of big fat cells (p= 0.035, 0.016, 0.046, 0.014 respectively). The vascular density was significantly higher in the fatty sections with big fat cells (p= 0.028). Inflammatory markers showed positive correlation with BMI and negative correlation with age. The vascular density and the distance to the synovial surface showed a trend of increasing in the young obese group. There was trend of increasing vascular wall thickness in the young obese group which was also observed in the correlation.

Conclusion: The synovium of the obese patients showed more inflammatory response but the vascularity was not significantly different. Increased fat cell size significantly attracted inflammatory cells. Vascular hyperplasia (increased wall thickness) is an indication of atherosclerosis in the synovial vessels.

DECLARATION

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

DECLARATION OF ORIGINALITY

This thesis is a product of my own work in collaboration with the Musculoskeletal Science Research Group and the Liverpool Musculoskeletal Biobank, produced during my time at the Department of Molecular and Clinical Cancer Medicine, University of Liverpool, between August 2014 and July 2017. The thesis was written by me with guidance from my supervisors Mr Joseph Alsousou, Dr Margaret Roebuck and Professor Simon Frostick.

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GLOSSARY OF ABBREVIATIONS

ADAMTS	A Disintegrin and Metalloproteinase with Thrombospondin motifs
ANOVA	Analysis of Variance
APCs	Antigen Presenting Cells
BMI	Body Mass Index
CD	Cluster of Differentiation
COX	Cyclooxygenase
DAB	3,3'-diaminobenzidine
DAMPs	Damage-Associated Molecular Patterns
DC	Dendritic Cell
dH_2O	Distilled Water
ECM	Extra cellular matrix
FFPE	Formalin-Fixed, Paraffin-Embedded
HRP	Horseradish Peroxidase
IHC	Immunohistochemistry
IGF-	Insulin Growth Factor -
IL-	Interleukin -
IMS	Industrial Methylated Spirits
IPFP	Infra Patellar Fat Pad
kDa	Kilodalton
KL	Kellgren-Lawrence
LMB	Liverpool Musculoskeletal Biobank

MCS	Mental Component Summary
MetS	Metabolic Syndrome
MoAb	Monoclonal Antibody
MMP	Matrix metalloproteinase
NHS	National Health Service
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
OA	Osteoarthritis
PAMPs	Pathogen-Associated Molecular Patterns
PCS	Physical Component Summary
PIS	Patient Information Sheet
PROMs	Patient Reported Outcome Measures
PRRs	Pattern Recognition Receptors
RA	Rheumatoid arthritis
REC	Research Ethics Committee
ROS	Reactive Oxygen Species
SF-12	12-item Short-Form Health Survey
SOPs	Standard Operating Procedures
SPSS	Statistical Package for Social Sciences
TGF-	Transforming Growth Factor -
TNF-α	Tumour necrotic factor - alpha
WHO	World Health Organisation
WOMAC	Western Ontario and McMaster Universities Osteoarthritis
WPBs	Weibel-Palade bodies

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1. INTRODUCTION

Osteoarthritis (OA) is a very common musculoskeletal disorder typically affecting older people and leading to disabilities. It is the most common form of arthritis. OA is not a single condition or a disease but rather a common complex disorder with multiple risk factors. The risk factors are broadly divided into genetic factors, constitutional factors and local factors (National Clinical Guideline, 2014). These can also be considered as modifiable and non-modifiable factors. The modifiable factors include obesity, lifestyle, repetitive micro-trauma and joint surgery (Michael *et al.*, 2010, Heidari, 2011). Although OA is linked to old age, young people can also get it. It is usually secondary to trauma and multiple injuries to the joints. Primary OA can also affect young people and this is seen more in obese individuals (Blagojevic *et al.*, 2010).

Obesity has become a pandemic with many effects on health and the economy (Meldrum *et al.*, 2017). Obesity has also been implicated in the early development of OA. It has been investigated extensively and multiple theories have been proposed on the aetiology of OA in this group, which are mainly divided into either mechanical or systemic causes. The evidence in favour of mechanical causes suggests that obese individuals are more than twice as likely as non-obese people to develop OA (Blagojevic *et al.*, 2010). One pound of weight loss unloads 4 pounds of joint stress in people with knee OA, leading to reduced pain (Messier *et al.*, 2005). Similarly, a loss of as little as 11 pounds in women reduced the incidence of OA by half (Felson and Zhang, 1998). Moreover, obese patients have greater sagittal-plane knee moments and greater knee joint load than normal weight adults (Browning and Kram, 2007).

Furthermore, a longitudinal study conducted in Michigan from 1962 until 1985 showed that obesity was significantly associated with the 23-year incidence of OA in the hands among subjects who were disease free at baseline. Greater baseline weight was also associated with greater subsequent severity of OA of the hands (Carman *et al.*, 1994). In the US National Health and Nutrition Examination Survey (NHANES), wave III (1988–1994), data from over 7,000 adults suggested that each

component of metabolic syndrome (the medical term for a combination of diabetes, high blood pressure and obesity) was more prevalent in people with OA (Puenpatom and Victor, 2009). Moreover, the histological characteristics of the synovium (the membrane lining the synovial joint) taken from patients with OA and those with mild RA were similar (Haraoui *et al.*, 1991). All these factors suggest a systemic cause of OA, which is chronic inflammation associated with obesity, referred to as metabolic OA. In the knee joint, the infrapatellar fat pad (a collection of fat behind the patellar tendon and below the patella inside the knee joint) is an established source of adipokines, which are capable of modulating inflammatory and destructive responses in knee OA through low-grade inflammation (Goldring and Otero, 2011, Clockaerts *et al.*, 2010). At the molecular level, the fat tissues in obese patients release adipokines (cell signalling proteins secreted by adipose tissue), which are recognised by the innate immune system and can initiate a pro-inflammatory response. Excess fat leads to activation of macrophages to change to M1 phenotype, which is pro-inflammatory in nature (Kanneganti and Dixit, 2012).

Despite numerous studies looking into obesity and OA, the knee joint synovium of young obese people has not been examined thoroughly before. It would be interesting to know if there is a difference between the synovium of young obese and lean people. This topic attracts several questions related to obesity and OA. For example, why do young obese people get early OA whilst old lean people present with OA very late? Are there any differences in their synovium that make one group do better than the other? How is inflammation related to the local fat burden?

To address these questions, multiple Immunohistochemical factors have been examined in this work. Immunohistochemistry was used to examine the synovium of young obese, young lean and old lean people to look at angiogenesis, inflammatory cells and synovitis. Angiogenesis was examined as overall vascularity using CD34 and activated vessels using von Willebrand Factor. One marker was used to quantify each inflammatory cell: CD3 for T cells, CD20 for B cells, CD68 for macrophages and vimentin for fibroblasts.

The complexity of conducting this research was obvious from the start as we were dealing with a diverse group of patients with different backgrounds and possibly different causes of OA. A lot of attention was placed on matching all the groups to be able to reach conclusions regarding the differences.

These markers were studied in detail to identify differences and correlations between groups in relation to age, body mass index (BMI) and fat cell diameter.

2. LITERATURE REVIEW

2.1 Osteoarthritis (OA)

OA is a very common musculoskeletal condition. In the UK, about 8.75 million people (33%) aged 45 and above have sought treatment for OA and 60% of them were females, according to The State of Musculoskeletal Health 2017 (Arthritis Research UK) (UK, 2017). In the US, it is estimated that nearly 27 million people have clinical OA (an increase from the estimate of 21 million in 1995) (Lawrence *et al.*, 2008). This shows the magnitude of the problem of OA, which is continuing to grow, especially with the increased life expectancy. There is no doubt that this will lead to a considerable cost and burden to the affected individuals and the state. Data from Schofield and colleagues showed that the income of people have retired owing to arthritis is five times less than that of those who are still working. Furthermore, the loss of workforce productivity, lost income tax revenue and increased government support to these individuals cost the state considerable amount of money (Schofield *et al.*, 2013). In developing countries and poor nations, where patients do not have access to treatments such as joint replacements, the impact of arthritis is significant, placing a tremendous burden on communities (Fejer and Ruhe, 2012).

2.1.2 Pathophysiology

OA is characterised by progressive loss of articular cartilage and the formation of osteophytes, which lead to chronic pain and functional restrictions in the affected joints. It involves matrix fibrillation, fissure appearance, gross ulceration and full thickness loss from the joint surface (Martel-Pelletier, 2004). The exact mechanism of cartilage breakdown in OA is still not very clear. It is a complex interaction of a multitude of factors, including genetic, environmental, metabolic and biochemical and mechanical factors (Sandell and Aigner, 2001, Kapoor *et al.*, 2011, Loeser *et al.*, 2012). The pathophysiological process of OA can be divided into three overlapping stages, as summarised by Martinek: the first stage starts at the molecular level with degradation of the matrix network. The size of aggrecan (a matrix molecule) decreases and the water content increases, which affect the mechanical properties

of the articular cartilage, making it less stiff. As a result, in the second stage, chondrocytes try to compensate by increased proliferation and metabolic activity. This condition can remain for several years. Finally, in stage three, the chondrocytes are not able to keep up their repair activity and a complete loss of cartilage tissue occurs as a consequence (Martinek, 2003).

A multitude of risk factors lead to OA development, which can be divided into controllable and uncontrollable factors. The former include trauma, repetitive micro-trauma, overweight and obesity, joint surgery, such as meniscectomy, bone density, muscle weakness and lifestyle. The uncontrollable factors include age, genetics, gender, hormonal changes and ethnicity (Michael *et al.*, 2010, Heidari, 2011). These factors work together to determine the risk for OA.

OA was thought to be a wear and tear problem originating in the articular cartilage as a consequence of either any process that would cause an abnormal load on any particular joint or owing to fragility and degeneration of articular cartilage. This paradigm changed with the advent of molecular biology and the discovery of many soluble mediators, such as cytokines, that can increase the production of matrix metalloproteinases (MMP) by chondrocytes (Berenbaum, 2013). This has led to the theory of inflammation as a cause of OA.

One of the obvious signs of an inflammatory process is the presence of synovitis and joint effusion, a feature linked to inflammatory arthropathies like rheumatoid arthritis. Synovitis has been observed in early- and end-stage OA and involves infiltration of mononuclear cells into the synovial membrane and production of pro-inflammatory mediators, including interleukin 1β (IL-1β), tumour necrosis factor-α (TNF-α) and chemokines. Other inflammatory markers have been observed in the synovial fluid but with lower abundance when compared to in RA patients. They are MMP-1, MMP-3 and MMP-13, cysteine cathepsins B and S, as well as IL-6, which reflect the importance of inflammation as a cause of arthritis (Pozgan *et al.*, 2010). Synovitis is observed in OA during flare-ups of the condition. The inflamed synovium produces pro-inflammatory mediators such as nitric oxide, prostaglandin,

neuropeptides and cytokines, which cause increased production of the proteolytic enzymes responsible for cartilage breakdown. This in turn amplifies synovial inflammation, creating a vicious circle (Sellam and Berenbaum, 2010). It was found that high sensitivity C-reactive protein levels reflect synovial inflammation and are associated with pain level (Pearle *et al.*, 2007). The reason that the synovium becomes inflamed in OA is still under debate. It has been hypothesised that the trigger is a cartilage fragment that acts as a foreign body, leading to activation of the synovium to produce pro-inflammatory mediators that then activate the chondrocytes to produce the degrading enzymes, which will further breakdown the cartilage (Sellam and Berenbaum, 2010). Another hypothesis suggests that the problem originates in the synovium, as evidenced by the link of macrophages in OA where macrophage-depleted synovium did not generate MMP-induced neoepitopes in a mouse experiment (Mapp and Walsh, 2012). It is difficult to know for certain where OA starts in the joint. However, it is important to examine the joint in a holistic manner and look at it as an organ with multiple parts that interact with each other to maintain function.

The innate immune system (see the inflammation section) was found to play a role in OA. A study has shown that TLR levels are increased in OA cartilage lesions. Other TLRs, including low molecular weight hyaluronic acid, fibronectin, tenascin-C and alarmins (S100 proteins, high-mobility group protein B1 [HMGB1]), have been found in OA synovial fluid. They are known to induce inflammatory responses. S100 proteins are involved in synovial activation and cartilage destruction (Kim *et al.*, 2006, van Lent *et al.*, 2012). Many other studies have shown links between the innate immune system and OA (Nair *et al.*, 2012, Wang *et al.*, 2011, Rosenthal, 2011).

Another theory of OA is the low-grade inflammation has been proposed based on an interesting study that looked into the gene expression profiles in peripheral blood leukocytes from patients with OA compared with a non-OA control group. There were two distinct subgroups of peripheral blood leukocytes in the OA group (Attur *et al.*, 2011). In the same study overexpression of IL-1β was associated with

higher pain scores and increased risk of OA progression. This leads us to obesity as a risk factor for OA owing to the chronic low-grade inflammation. However, mechanical load also has been implicated in obesity.

2.1.3 Obesity and OA

Obesity has received significant attention as an important risk factor for OA development, especially after the identification of adipose tissue as an endocrine organ that is able to produce biologically active substances called adipokines (Goldring and Otero, 2011). The infrapatellar fat pad is an established source of adipokines and is capable of modulating inflammatory and destructive responses in knee OA. Low-grade inflammation has been attributed to circulating adipokines (Clockaerts *et al.*, 2010). In the same study by Clockaerts and colleagues, the infrapatellar fat pad was found to contain macrophages, lymphocytes, granulocytes and nociceptive nerve fibres, which could in part be responsible for anterior pain in knee OA. In addition, the infrapatellar fat pad secretes cytokines, interleukins, growth factors and adipokines that influence cartilage by up-regulating the production of MMPs, stimulating the expression of pro-inflammatory cytokines and inhibiting the production of cartilage matrix proteins. The end result is cartilage degradation. Although adipose tissue could cause a low-grade inflammatory process, other mechanisms may also contribute to OA in obese people. Along with the loss of muscle mass and strength over time, obesity is associated with mechanical stress. All these possible pathways could propagate OA (Vincent *et al.*, 2012).

Mechanical load and movement are essential to the survival of the chondrocytes. Based on *in vitro* mechanical loading experiments, injurious static compression stimulates the depletion of proteoglycans and damage to the collagen network, and decreases the synthesis of cartilage matrix proteins. On the other hand, dynamic cyclic compression increases matrix synthetic activity (Torzilli *et al.*, 2010). Impact injury can lead to chondrocyte death through the release of reactive oxygen species (ROS), which are responsible for up regulating MMP-13, ADAMTS-5, and TNF-α (Ding *et al.*, 2010). One could argue that in obese people, cyclical load is taking place,

which would help maintain the articular structure. It is difficult to assume this as the mechanics of the joints can also be affected, leading to abnormal cyclical loading. It is also worth noting that weight loss improves OA symptoms (Messier *et al.*, 2005). Weight reduction reduces pain and increases physical function (Messier *et al.*, 2000, Christensen *et al.*, 2007). It was found that compressive forces through load-bearing joints such as the knee are reduced by almost fourfold due to weight loss (Messier *et al.*, 2005). There is no doubt that obesity is an important risk factor for OA and it involves complex interconnected pathways. Moreover, obesity is a component of metabolic syndrome. Metabolic syndrome (MetS) describes a number of related conditions, such as insulin resistance, visceral obesity, dyslipidaemia and hypertension, which on their own significantly increases a patient's risk of cardiovascular disease. People with metabolic syndrome are at a higher risk of having OA (Puenpatom and Victor, 2009). OA that is related to obesity and metabolic syndrome is called metabolic OA and is considered as a subtype of OA. In fact, metabolic OA has been proposed to be the fifth component of metabolic syndrome (Zhuo *et al.*, 2012).

2.1.4 Smoking and OA

In a recent article by Dube *et al*. (Dube et al., 2016), the negative effect of smoking on OA was summarised in five possible mechanisms: 1) Effects of smoking on cartilage loss as evidenced by knee MRI findings that showed a significant association between cartilage loss and pack-year smoked (Davies-Tuck *et al.*, 2009). 2) Smoking triggers genetic predisposition to OA as shown by *Ding et al.* (Ding et al., 2007), where MRI showed reduction in the cartilage volume in people who smoke and have a family history of OA a study that shows gene-environment interaction in the aetiology of knee OA. 3) Smoking's effects on inflammation are very well established and profound. Cigarette smoke produces thousands of ROS, which has a significant impact on the immune system and activation of inflammation (Lee *et al.*, 2012, Huang *et al.*, 2005). 4) Smoking is also associated with metabolic syndrome and both insulin resistance and higher BMI. The risk of metabolic syndrome reduces upon smoking cessation (Chiolero *et al.*, 2008).

2.2 Anatomy
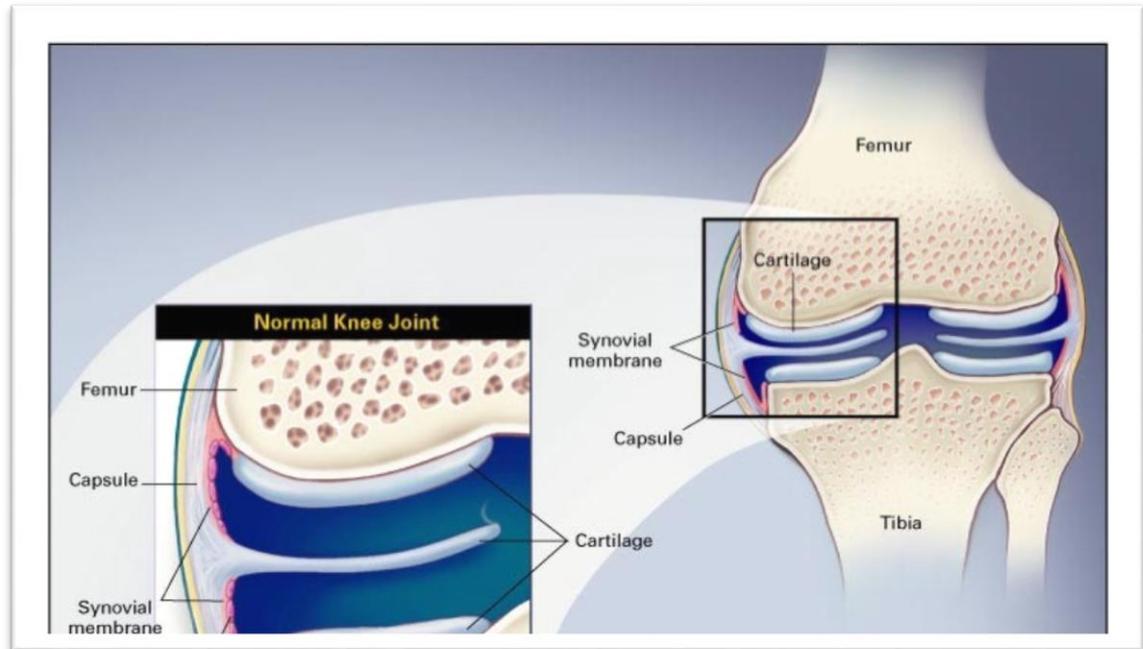
Figure 2. 1: Anatomy of the knee joint showing the synovial layer, adapted from(Choy and Panayi, 2001)

Synovial joints are unique structures. They are lubricated connections between bones and are the reason for our flexibility. They are fluid filled cavities separating layers of hyaline articular cartilage. It is suggested that they first evolved as vertebrates moved onto land (Askary *et al.*, 2016). There are different hypotheses as to where they first evolved and the prevailing hypothesis is that lubricated synovial joints first evolved in tetrapods in response to newfound mechanical challenges imposed on the weight-bearing joints of nascent limbs (van der Kraan, 2013a, van der Kraan, 2013b). An old histological study suggested that jaw joints of lungfish have a synovial-like morphology (Bemis, 1986). The latest findings suggest that they evolved even earlier than what is currently accepted (Askary *et al.*, 2016).

The knee joint is a modified hinge joint that allows flexion, extension and rotation, yet provides complete stability and control over a wide range of loading conditions (Goldblatt and Richmond, 2003). The stability is derived from the shape of

the bone as well as the surrounding soft tissues, such as muscles and ligaments. Within the knee joint, there is specialised membrane (synovium) that lines the inside of the knee joint and provides it with the synovial fluid that is essential in the knee mechanics and the viability of the articular cartilage. In general, the term synovium refers to the soft tissue lining the spaces of diarthrodial joints, tendon sheaths and bursae (Smith, 2011). It includes the continuous superficial layer of cells (intima) and the underlying tissue (subintima). The intima consists of macrophages and fibroblasts while the subintima includes blood and lymphatic vessels, a cellular content of both resident fibroblasts and infiltrating cells in a collagenous extracellular matrix (Smith, 2011). The normal synovial membrane is stated to be a relatively acellular structure consisting of a distinct intimal lining layer of one to two cells thickness and a synovial sublining layer. The latter is relatively acellular, containing scattered blood vessels, fat cells and fibroblasts, with few lymphocytes or macrophages. Typically, the normal synovium contains an intimal layer of 20–40 µm thickness in cross-section and an areolar subintima, which can be up to 5 mm in thickness.

The normal synovial tissue can be divided into three different categories microscopically. This depends on the structure and content of the subintimal layer. It can be fibrous, areolar and adipose. In some areas of the body, the subintima may consist of periosteum, perimysium or even hyaline or fibrocartilage. The areolar type of normal synovium is the most specialized form; most of the time it is folded and can unfold when stretched. There is usually a continuous layer of lining cells, often two or three deep, on the tissue surface (called the intima) (Smith *et al.*, 2003, Smith, 2011). Immediately beneath the intimal layer are capillaries, with a deeper plexus of small arterioles and venules, associated with mast cells (Davies, 1950, Wilkinson and Edwards, 1989). Lymphatic vessels are present in all types of normal synovial tissue and are usually found in the deep subintima. The extracellular matrix of normal synovium has different consistencies in different layers. Type I collagen is scant in the intimal layer and more abundant in the subintimal layer. The deepest area of the normal synovium consists of a loose connective tissue layer, which allows

the membrane to move freely. Below this loose layer there will often be found ligament, tendon or periosteum (Smith, 2011).

Electron microscopy of the synovium by Barland and colleagues (Barland et al., 1962) showed that the lining cells are divided into two cell types or states of activity on the basis of their cytoplasmic contents. Type A synoviocytes with a phagocytic function and type B synoviocytes, which are responsible for secretory functions in the synovium. The former can be found in the macrophage lineage and the latter in the fibroblast lineage.

In normal synovium, macrophages make up a minority of cells, while the numbers increase significantly in inflammatory arthritis. For example, in RA synovial tissue, macrophages account for up to 80% of the intimal layer. The usual pattern is that of a superficial layer of macrophages with an intimal phenotype, below which is a layer of intimal fibroblasts. In OA synovium, the inflammatory cells are also more especially macrophages (Farahat *et al.*, 1993). There is good evidence to suggest that the macrophages in the synovium originate from circulating monocytes, which come from the bone marrow.

Fibroblasts (Type B synoviocytes), have adapted to the production of hyaluronan (Wilkinson *et al.*, 1992). Synovial fibroblasts are different from the normal population of fibroblasts as they express adhesion molecules that are not seen in the normal population. These are thought to be important in cellular trafficking as they trap macrophages and lymphocytes in the synovium but allow neutrophils to egress to the synovial fluid (Edwards *et al.*, 1997). Other types of cells are found in the synovium, including T and B lymphocytes, which are thought to play a role in trafficking in the normal synovium. Dendritic cells are also found in the synovium (Smith, 2011).

The extracellular matrix in the intimal layer is composed of a variety of fine fibrillar ultrastructures, including collagens III, IV, V and VI with a little type I collagen. There are other structures in the intima, including laminin, fibronectin and chondroitin-6-sulfate-rich proteoglycan, which are all components of the basement

membrane (along with collagen IV), but there is no basement membrane beneath the intimal layer in normal synovium (Revell *et al.*, 1995).

The vasculature of the synovium is unique with some fenestrated capillaries located just beneath or within the intima. This type of vascular anatomy allows for the communication between the immune system with the synovial joint. The vascularity increases in the deeper layer. In the subintimal layer, there are larger venules, arterioles and lymphatics. They form an anastomosing array (Xu *et al.*, 2003). Capillaries are very prominent in inflammatory arthropathies such as RA. Essentially, any inflammatory process will cause proliferation of endothelial cells, which is addressed in this research in relation to fat levels.

The functions of normal synovium are: providing a deformable structure to allow movement of nearby relatively non-deformable tissue structures because of the specialised viscoelastic characteristics of materials like collagens in the synovium, maintenance of an intact non-adherent tissue surface, joint lubrication, control of synovial fluid volume and composition, and chondrocytes. The non-adherent tissue surface is achieved by the production of hyaluronan by the intimal B synoviocytes. The latter cells also produce plasminogen activator and decay accelerating factor (DAF), which may inhibit fibrin formation and scarring. The maintenance of the synovial fluid is a delicate process where there is a free exchange of crystalloids and proteins, and inhibition of the rapid transit of viscous hyaluronan solution, which is an important component of the fluid. This is achieved by intimal fibroblasts and macrophages. The unique structure of the vascularity also plays a role in getting the maintenance of this fluid through recruitment of inflammatory cells such as macrophages from the blood monocytes.

The lubrication of the synovial joint is an important function of the synovial fluid to enable long-lasting, functioning frictionless joints. The size-selective semi-permeable membrane of the synovium is an important part of the synovial fluid formation. The synovial fluid (SF) is a dialysate of blood plasma with contribution from the molecules secreted by the cells lining and within the synovial tissue. It contains lubricant molecules, namely hyaluronan and proteoglycan 4, also known as

lubricin, and superficial zone protein (Schumacher *et al.*, 1994, Jay *et al.*, 2000, Hui *et al.*, 2012). These are high molecular weight molecules and are trapped in the synovial joint while low molecular weight molecules can leave the join, including most metabolic substrates and byproducts, cytokines and growth factors. The synovial membrane also acts as a barrier against high molecular weight plasma molecules entering the joint. In the disease process, this mechanism of strict control does not function properly, leading to alteration of the synovial fluid contents and characteristics (Hui *et al.*, 2012).

Synovial fluid is a viscous, non-Newtonian fluid (*i.e.* shear stress is reduced with increased shear rate). Thus, the SF behaves as a viscous material at low rate of oscillation and as elastic martial at high frequencies. This is also called shear-thinning behaviour and occurs in normal and OA synovial fluid. These properties are mainly owing to the concentration and molecular mass of hyaluronan (Schurz and Ribitsch, 1987). On the other hand, SF in RA exhibits a Newtonian behaviour.

2.2.1 Articular cartilage

Although this research is mainly focused on the synovium, the anatomy of the articular cartilage has to be mentioned to better understand the structure. The articular cartilage is a unique structure that has a smooth surface. It is avascular, aneural, alymphatic and almost non-immunogenic. It receives its nutrition from the synovial fluid circulating in the joint. It is a composite material with cells embedded in an extracellular matrix. Chondrocytes represent the cellular component of the articular cartilage. The extracellular matrix (ECM) is composed of fibres and ground substance. Collagen, especially type II, is the main fibre, representing about 10–20% of the wet weight. Other fibres such as elastin are also present. The major constituent of the ECM is water (representing about 75% of the wet weight). Ground substance includes water, proteoglycans and glycosaminoglycans, glycoproteins, degradative enzymes like MMPs and extracellular ions.

The articular structure can be divided into four layers histologically. These layers are: the superficial layer (gliding zone), the middle layer (transitional zone), the deep layer (radial zone) and the calcified layer (calcified zone). The arrangement of the collagen fibres is different in each zone to adapt to the function of the joint during movement. The fibres are parallel to the articular surface in the superficial zone to resist shear stress. This arrangement changes gradually from parallel to oblique in the transitional zone to end up in a perpendicular direction to the articular surface. The latter helps to resist compression forces (Ramachandran, 2007).

As there is no blood supply to the articular cartilage, self-renewal is limited to poor quality regeneration, which lacks the capacity to withstand normal physiological stresses (Lorenz and Richter, 2006). Although the avascular feature of the articular cartilage seems to be a disadvantage for cartilage repair, it is actually a very intelligent design. If we imagine, for the sake of argument, that the articular cartilage is full of blood vessels, this will put it at risk of bleeding with any physiological load. This, of course, is not a good idea as we know that bleeding in the joint will lead to early OA, which is very well documented in cases of haemophilia. To avoid bleeding, the vessels have to be protected with something hard, like what happens in the bone, but this will defeat the purpose of the smooth and soft articular cartilage. Thus, the articular cartilage relies on the neighbouring synovium to do the job of nutrition through the synovial fluid. The synovial fluid brings nutrients and components of cellular repair to the chondrocytes by diffusion. The chondrocytes are metabolically very active, but normally do not divide after adolescence. They can repair only small defects associated with minimal loss of matrix components. More extensive defects will result in permanent damage to the articular surface (Wollheim, 1999).

2.3 Microcirculation

2.3.1 Basic vascular anatomy

Microcirculation refers to the vascular bed where vessel diameters are smaller than 100 µm. They are designed to reach tissues at the cellular level to transport oxygen and nutrients and remove cellular waste (das Gracas Coelho de Souza *et al.*, 2016). They are very sensitive to changes in systemic blood pressure, which is readjusted by the vascular tone to meet the local oxygen requirements. The blood vessels exhibit dynamic changes to adapt to the physiological demand. Their lumen is covered by endothelium, which is formed by a monolayer of endothelial cells (ECs). The ECs are very important to the physiological changes that happen to the local microcirculation. Their position between the blood stream and the vascular smooth muscle cells (VSMCs) makes them capable of sensing physical and chemical stimuli from either side of the lumen (Deanfield *et al.*, 2007, das Gracas Coelho de Souza *et al.*, 2016). The microvessels are subdivided into arterioles, capillaries and venules. Arterioles are the start of the microcirculation system and are responsible for controlling the blood flow to the tissue. The arteriolar ECs are surrounded by VSMCs, which contract and relax in response to the vasoactive factors released by the nervous system (den Uil *et al.*, 2008). The arterioles continue as capillaries, which are formed by one EC layer surrounded by a basement membrane. It is this anatomical feature that allows them to carry out the exchange of molecules between blood and tissue. The lumen diameter is about 5 µm. Finally, the capillaries end up as venules, which join the bigger veins. Thus, for the vessel to qualify for the function of exchange of molecules, it should not contain a smooth muscle layer (den Uil *et al.*, 2008). Figure 2.1 shows the details of the anatomy.
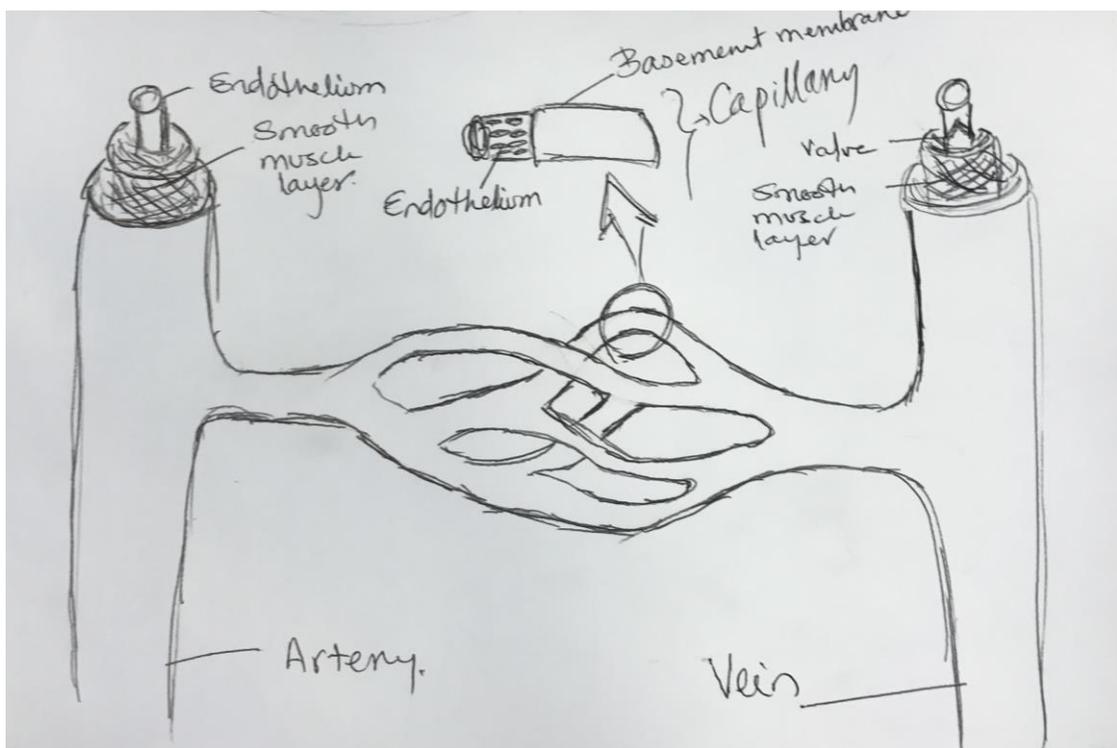
Figure 2. 2*: Anatomy of microcirculation*

2.3.2 Microcirculation in the synovium

This has been discussed briefly in the anatomy section (section 2.2). Levick (Levick, 1995)explained the details of the synovial microcirculation and how it is related to the actual function in normal and abnormal conditions. The synovium has a very rich microvascular bed composed of capillaries and post-capillary venules. In a normal human knee synovium, the vascular density average 240 mm^{-2}. These capillaries are very close to the synovial surface. In normal individuals the average distance is 35 µm but this distance is more than double in RA. The average transport distance in chronic RA is more than twofold that of the normal synovium and the density is reduced by one third. About 50% of the capillaries in the synovium are fenestrated and the rest are continuous. The fenestration is usually facing the synovial surface. All these features are essential to facilitate the exchange of fluid and to provide the chondrocytes with nutrition.

2.3.3 Adipose tissue and microcirculation

Adipose tissue is considered as an endocrine organ that secretes a lot of adipokines, which are capable of inducing inflammation. The excessive accumulation of adipose tissue noted in obesity leads to adipocyte hypertrophy, which results in a decrease in the vascular density and hence the blood flow, which will eventually lead to adipose tissue hypoxia. As a result, adipocyte necrosis will occur, which potentiates an inflammatory response (Ouchi *et al.*, 2011). This inflammatory response will result in atherosclerosis, which will further worsen the local blood supply. It is interesting to know that weight loss in obese people will lead to an improvement in the structure of the vessels (De Ciuceis *et al.*, 2011). Although atherosclerosis affects blood vessels systemically, local factors can play a role. All blood vessels except the cerebral vasculature have an outer fatty layer called adventitia. It is also called perivascular fat or perivascular adipose tissue (PVAT)(Gao, 2007). It was believed that PVAT provides only structural support to the vessels. However, in cases of obesity, vascular injury, aging and infection, the PVAT causes adipocyte abnormality and activation of inflammatory response, which lead to VSMC growth and proliferation, which, in turn, can cause vascular disease including atherosclerosis, restenosis and hypertension (Miao and Li, 2012). VSMCs can also be modulated by the endothelial cells and the nerve fibres (Feletou *et al.*, 2008).

2.4 Inflammation

Inflammation is a coordinated process induced by microbial infection or tissue injury (Nathan, 2002, Vinay Kumar, 9th Edition). It is a complex process whereby the body aims to repair itself relying on different mechanisms. There are two main systems that act harmonically to reach a state of homeostasis. The innate immune system is the first line of defence against any external or internal stimuli. It has a broad-spectrum function against internal and external injuries. It is believed to stimulate the adaptive immune system to target specific microbes and retains a memory of the pathogen to act faster when it recurs, but this adaptive system take longer to activate in the first instance (Alberts B, 2002).

2.4.1 The Innate immune system

The innate immune system is an ancient system that is present in all protozoa. As a first line of defence against all microorganisms and external injuries, it has a specific mechanism by which it can act quickly and recognise pathogens. This happens through pattern recognition receptors (PRR), which detect the pathogen-associated molecular patterns (PAMPs) exhibited by microorganisms (Medzhitov and Janeway, 2000). The PAMPs represent molecular structures that are produced only by the microbial pathogens, but not by the host organism. For example, the bacteria but not the eukaryotic hosts produce peptidoglycans and liposaccharides. This property of PAMPs accounts for self-/non-self-discrimination by the adaptive immune system of vertebrates as well. They represent conserved molecular patterns that are essential for the survival of the microbes. PAMPs are often shared by large groups of microorganisms, which allows the immune system to develop a limited number of germ line-encoded pattern recognition receptors to recognise the vast variety of microorganisms. The molecular structure of the PAMPs represents a signature of a pathogen class that allows the immune system to choose the best defence mechanisms against a given class (Medzhitov and Janeway, 2000, Medzhitov, 2007).

PRRs are germline-encoded receptors evolved in host organisms to recognise PAMPs. They are heterogeneous proteins with different domains involved in pattern recognition (Medzhitov and Janeway, 2000). They can be involved in: 1) opsonisation of bacteria and viruses for phagocytosis or activation of the lectin pathway of complement (Fraser *et al.*, 1998), 2) uptake of pathogens by phagocytes and dendritic cells (Stahl and Ezekowitz, 1998), or 3) triggering of the signalling pathways that result in the induction of transcription of a variety of immune response genes.

The body has to distinguish between self and non-self, and starts an inflammatory response to suit the situation. Inflammation can be triggered by infection, trauma, post-ischemic injury, toxins and autoimmune injury. The innate immune system is activated after such injury either through the PAMPs or other

factors that are released following trauma, such as heat shock proteins, HMGB1, neuropeptides and mast cell contents, including histamine, leukotrienes, PGD2, tryptases chemokines and TNF. The immune system has evolved to control infections or any other injury in a rapid way but alongside the control of the infection or injury there is also damage to local tissue by the inflammatory response. Thus, the immune system has to distinguish between infection and a simple trauma and then has to decide how bad the injury is or whether there is a combination of both infection and trauma. The immune system has to act quickly and control the injury as soon as it gets activated by the factors listed above. This happens through recruitment of leukocytes from the blood (Muller, 2003). However, to act fast the leukocytes should be residing in the tissue. Mast cells and macrophages fulfil this function. Once an injury occurs, the mast cell releases its content with all the pro-inflammatory factors and starts activating other inflammatory cells. The first cell to arrive to the injury site is the neutrophil, which further amplifies the inflammatory response by activating the mast cells and also releasing other chemotactic agents to recruit more inflammatory cells. The neutrophils undergo respiration, where they release proteinases, other hydrolases and oxidants. This action leads to tissue breakdown. The macrophages are activated and also help in the amplification of the inflammatory response. The antigen-presenting cells are attracted to the site of injury or infection, and in turn activate lymphocytes to act in a more targeted way against specific pathogens, leading to less unnecessary damage to the surrounding tissue.

Accordingly, there are four distinct phases of the inflammatory cascade: recognition of infection using the aforementioned mechanisms by the PRRs, recruitment of inflammatory cells to the site of infection, elimination of the microbe, and resolution of inflammation and return to homeostasis (Vinay et al., 2015). One of the functions of the innate immune system is to activate the adaptive immune system to help in controlling the inflammatory cascade.

2.4.2 The adaptive immune system

The two main cellular components of the adaptive immune system, T and B cells, both play major roles in protective immunity against infectious pathogens and have the potential to eradicate cancerous cells (den Haan *et al.*, 2014). It is the function of the innate immune system to activate this highly specific system to aid in eradicating any infectious focus, although it is not always necessary to activate the innate immune system to activate the adaptive system (Alberts B, 2002).

The T and B cells are activated by professional antigen-presenting cells, such as dendritic cells (DCs). The classical antigen-presenting cells are DCs, macrophages, Langerhans cells and B cells (Wollenberg and Bieber, 2002). In recent years, DCs have been found to regulate the cross talk between the innate and the adaptive immune system (Boltjes and van Wijk, 2014). Out of all the antigen-presenting cells, the DCs are the most potent in promoting activation of naïve T cells (Boltjes and van Wijk, 2014). The understanding of DCs has evolved since 1968 when studies Mosier and Coppleson showed that accessory adherent cells were required for *in vitro* immune responses (Mosier and Coppleson, 1968). It was thought that these cells are macrophages, but they were later found to be DCs (Hoefsmit *et al.*, 1982). Gene expression analyses, lineage tracing and barcoding experiments further indicated their existence as a separate hematopoietic lineage (Schraml *et al.*, 2013, Naik *et al.*, 2013). These cells identify the antigen then process it and present it to the lymphocytes for degradation. This process has been established very well (Neefjes *et al.*, 2011, Vyas *et al.*, 2008). It is clear that in acute inflammation the innate immune system is activated very quickly to contain the infection through the aforementioned mechanisms. However, the immune system is still working even if there is no external stimulus or antigen. It works to keep the body safe even from itself. The metabolites of cell metabolism can be harmful and there has to be a mechanism by which the body can take care of them. In this case, it is very important to have antigen-presenting cells, especially the DCs that are spread all over the body, to keep the body safe, even in the steady state. This leads us to the theory of low-grade inflammation as an important cause of OA.

2.4.3 Inflammation and angiogenesis

Angiogenesis and inflammation are very closely related processes and have gained increasing attention in recent years. This link was observed a long time ago with the appearance of newly formed blood vessels in granulation tissue, and the dual functionality of angiogenic factors, *i.e.*, they exhibit both pro-inflammatory and pro-angiogenic effects (Granger and Senchenkova, 2010). Although the two processes are completely different, they potentiate each other.

In normal tissue, capillary growth and proliferation are rarely noticed except during acute injury and wound healing or cyclical events in the female reproductive cycle; with appropriate stimuli, the process of angiogenesis can be started. This process is controlled by different factors, which can promote or inhibit angiogenesis. Thus, there has to be a balance between the angiogenic and angiostatic factors to determine the existence and rate of blood vessel proliferation in a tissue. During the inflammatory process, the balance is in favour of angiogenesis. It is believed that the hypoxic state in inflammation acts as a stimulus for angiogenesis through the hypoxia-inducible factor signaling pathway, which elicits the transcription-dependent production of VEGF and FGF. In addition, inflammation is also associated with the recruitment of circulating leukocytes and platelets, and the activation of resident macrophages, mast cells and fibroblasts, all of which are capable of producing large quantities of pro-angiogenic factors, including VEGF and cytokines (Naldini and Carraro, 2005, Lingen, 2001).

Angiogenesis relies mainly on the vascular endothelial growth factor (VEGF) family and its receptors (VEGFR-1 and VEGFR-2) for its regulation. Under direct stimulation from VEGF-A, the endothelial cells start proliferating, a process that requires engagement of VEGFR-2 with VEGF-A to activate tyrosine kinase, leading to sprouting of new vessels from existing microvessels. A prerequisite for sprouting is destabilisation of existing microvessels, which includes pericyte dropout, diminished cell–cell adhesion and dissolution of the basement membrane. So, the endothelial cell starts to leave the endothelial monolayer by penetrating through the basement membrane and invading the nearby interstitial space. The cell retains a specific

phenotype to enable it to produce lamellopodia. The latter extend ahead of the cell to sense the environment and determine the appropriate migratory direction. This cell acts as a foundation and is followed by migratory/proliferative cells, which allow for extension of the sprouting vessel and lumen formation. VEGF plays a major role in the process of sprout formation. The majority of the angiogenic effects of VEGF are mediated through VEGFR-2 which, along with VEGFR-1 activation, are necessary for the hyper-permeability associated with this process. There is recent evidence suggesting that bone marrow-derived cells are implicated in the formation of new blood vessels in chronic inflammation (Chidlow *et al.*, 2007, Dvorak, 2005, Hicklin and Ellis, 2005).

Chronic inflammation and angiogenesis are two different processes that work hand in hand. The two processes are stimulated by hypoxia, leading to the accumulation of macrophages and other immune cells and an increase in growth factor production (Murdoch *et al.*, 2005). There is abundant evidence to support the contribution of these factors, which are released in the inflammatory process, to vascular endothelial cells (Philip *et al.*, 2004, Coussens and Werb, 2002, Nathan, 2002). In other words, angiogenesis potentiates inflammation by providing oxygen and nutrients to help in the cellular metabolism of the inflammatory cells. It starts with the production of nitric oxide (NO) by the inflammatory cells. NO is a potent inflammatory agent, which, along with other inflammatory cytokines, stimulates vessel dilatation and permeability to allow immune cells to escape to the injury site (Charo and Taubman, 2004). The inflammatory cells require adhesion molecules for them to escape the vessels to the injury site in a process involving cell rolling, tethering and firm adhesion to the vascular wall of the endothelial cells, and finally extruding out of the lumen. Different types of pro-inflammatory factors control the adhesion molecules. Once the inflammatory cells are activated and reach the battle field, they release angiogenic factors, such as VEGF, Ang, bFGF, HGF, PDGF, TGF-beta, TGF-alpha and many others, at the inflammatory site, which induce mitogenic and migratory effects in the endothelium (Lee, 2005). As mentioned above, hypoxia is one important feature of inflammation, inducing the up-regulation of hypoxia-inducible factors expression, which leads to transcription of several angiogenic genes

encoding for VEGF and other angiogenic factors (Coussens and Werb, 2002, Naldini and Carraro, 2005).

A primary event in inflammation is activation of nuclear factor kappa-B (NF-kB), a transcription factor, by the pro-inflammatory mediators. It is crucial for regulating gene expression of a wide range of genes important in cell survival, including MMPs, urokinase type plasminogen activator, V-CAM, I-CAM and E-selectin molecules.(Charo and Taubman, 2004, Charo and Ransohoff, 2006, Karin, 2006). The end result is a continuous production of inflammatory and angiogenic molecules and the activation of MMPs, which degrade the extracellular matrix. There is growing interest in chronic inflammation and angiogenesis as the number of pathologies involving them are increasing. OA is an example of such pathologies.

OA is the most common type of arthritis and is thought to be degenerative in nature (Creamer and Hochberg, 1997). The aetiology of OA is multifactorial; however, inflammation is increasingly considered as a cause of arthritis progression (Smith *et al.*, 1997, Benito *et al.*, 2005, Brooks, 2003, Myers *et al.*, 1990). The synovial membrane of OA showed inflammatory infiltrates primarily of T cells and monocytes in over 50% of patient sample's as shown by (Myers et al., 1990). There is also evidence of increased C-reactive protein in OA patient sera and correlated with OA progression. (Spector *et al.*, 1997). In addition, several pro-inflammatory cytokines and chemokines have been detected in the synovial infiltrates in OA, including IFN, IL-1, IL-6, IL-8, IL12, IL-17 and TNF-alpha (Smith *et al.*, 1997, Myers *et al.*, 1990). These pro-inflammatory molecules act on specific receptors present on ECs, cartilage and bone cells, leading to the production of MMPs and plasminogen activator, which as mentioned before degrade the ECM and impair the structure and function of the cartilage (Vergunst *et al.*, 2005).

2.5 IHC markers of interest

2.5.1 Endothelial cells

2.5.1.1 Von Willebrand factor (vWF)

vWF is a glycoprotein essential for haemostasis. It can result in bleeding disorder if deficient of defective (von Willebrand disease). The basic vWF monomer contains 2050 amino acids. This large multimeric glycoprotein is produced constitutively as ultra-large vWF (it is assembled from identical approximately 250 kDa subunits into disulfide-linked multimers that may be >20,000 kDa.) in the endothelium and specifically in the Weibel–Palade bodies within the endothelial cells. It is also produced by megakaryocytes (α-granules of platelets) and sub-endothelial connective tissue (Sadler, 1998).

To understand vWF and its relation in our study, it is essential to understand the inflammatory process and what happens at the molecular level with the endothelial cells. Endothelial cells are important in monitoring the inflammatory response as they respond to changes in mechanical and chemical stress. The endothelial cell contains unique organelles called Weibel–Palade bodies (WPBs) in which the vWF is synthesised and stored. In fact, 95% of the contents of the WPBs are made up of vWF and its precursor propolypeptide (Proregion) with small amounts of other adhesion and signalling molecules like P-Selectin and IL8. WPBs are rod-shaped organelles between 100 and 300 nm thick and can be up to 4 µm long with internal tubules (approximately 15 nm thick) running parallel to the long axis and are embedded in an electron-dense matrix. They are restricted to endothelial cells and it was believed that they have an important role in vascular physiology, which was proven after the discovery of vWF within the organelles (Weibel and Palade, 1964, Jaffe *et al.*, 1973).

The WPBs contain an important protein that is very critical to initiation of inflammation. This protein is P-Selectin, which is a membrane protein of the WPBs that is involved in the recruitment of leukocytes to the activated endothelial cells. Once stimulated, WPBs fuse with the plasma membrane and P-selectin appears on the cell surface within 20 min of stimulation. The P-Selectin tethers leukocytes to endothelial cells and subsequently rolling of leukocytes on endothelia, a step that is essential in cell trafficking and initiation of inflammation (Hannah *et al.*, 2002).

Moreover, upon stimulation of endothelial cells, the contents of the WPBs (*i.e.* vWF) are secreted, which mediates the adhesion of platelets to sites of vascular damage by binding to specific platelet membrane glycoproteins and to constituents of exposed connective tissue. It is the size of vWF that makes it suitable for this function. VWF is also a carrier protein for blood clotting factor VIII, and this interaction is required for normal factor VIII survival in the circulation and hence haemostasis (Hannah *et al.*, 2002).

WPBs exocytosis is a complex process. It involves some form of fusion of the organelles with the plasma membrane that allows the release of the contents. There are two types of fusion: kiss and run fusion, in which case the fusion pores open and close rapidly, and a lingering kiss that allows the fusion pore to remain open for a longer period of time. The type of fusion that happens in the case of WPB is more of a lingering kiss. It is interesting to know that the fusion pores formed during the lingering kiss can act as filters that retain bigger molecules, like vWF, and release others. The lingering kiss events are proportional to the strength of the stimulus (Babich *et al.*, 2008).

The above evidence suggests that the vWF is very specific to endothelial cells and is a very specific marker to assess the activity of endothelial cells and also the amount of inflammation associated with it. In our research, we will look into this marker and carry out a quantitative assessment of the activated endothelial cells, and then relate it to the inflammatory process in the tissue sections.

2.5.1.2 CD34

CD34 was first identified in hematopoietic progenitor cells as a transmembrane phosphoglycoprotein. It was described independently by (Civin et al., 1984, Tindle et al., 1985) as a surface protein that functions as a cell–cell adhesion molecule. It has been studied extensively and has been found to be expressed in cells other than stem cells. Although CD34 is considered predominantly as a marker of hematopoietic stem cells, it is now an established marker of several non-hematopoietic cell types, such as vascular endothelial cells, embryonic fibroblasts, multipotent mesenchymal

stromal cells, interstitial dendritic cells and epithelial progenitors. It belongs to a family of single-pass transmembrane sialomucin proteins (Sidney *et al.*, 2014). Despite extensive investigation, CD34's actual function is still not very clear. However, it has been linked to inhibition or facilitation of adhesion, cell proliferation and regulation of differentiation (Healy *et al.*, 1995, Nielsen and McNagny, 2009).

CD34 has been investigated for sensitivity and specificity in diagnosing vascular tumours and was found to be a reliable marker for assessing vascularity (Miettinen *et al.*, 1994, Fina *et al.*, 1990). Although CD34 is expressed mainly in the luminal membrane of cellular processes, it may also be expressed on the luminal membrane of cells at the tip of the vascular sprouts. Those endothelial cells with CD34 markers are thought to be quiescent and involved in migration and adhesion (Fina *et al.*, 1990). This implies that CD34 gives an overall assessment of the vascularity of a given tissue but it does provide information about the endothelial cell activity in terms of neo-angiogenesis. The main objective of this research is to explore the angiogenesis, and CD34 and vWF markers have been chosen for this purpose. The CD34 marker focuses on the overall vascularity whereas vWF assesses only activated endothelial cells.

2.5.2 Macrophages

2.5.2.1 CD68

Macrophages are white blood cells and are considered as one of the big eaters of the immune system. They are differentiated from the monocytes, which are in turn originated from the bone marrow progenitor cells. Macrophages live in every tissue of the body. In case of tissue damage or infection, monocytes are rapidly recruited to the tissue, where they differentiate into tissue macrophages. The role of macrophages in inflammation has been established and discussed earlier in the literature review (see inflammation section 2.4). Macrophages play an important role in the synovium as inflammatory cells that regulate inflammation. They are considered as a principal morphological characteristic of synovitis (Benito *et al.*, 2005). Benito and colleagues (Benito et al., 2005) studied the differences between early and late OA and found that synovial lining thickness and CD68 expression are

significantly higher in early OA. They control cytokines, which drive cartilage breakdown. Although other cells also produce these cytokines, such as chondrocytes and fibroblasts, macrophages are considered the key stimulator of these processes (Sun *et al.*, 2016). The role of macrophages in OA has been discussed in the section of pathogenesis of OA (section 2.1).

Macrophages have different phenotypes and functions and have the capacity to change phenotype depending on the local tissue circumstances; hence, they are referred to as heterogeneous cells. They are classified into M1 and M2. M1 macrophages are pro-inflammatory whereas M2 macrophages are anti-inflammatory (Liu *et al.*, 2014).

CD68 was used as a classic marker of macrophages in the synovial tissue. 'This gene encodes a 110-kD transmembrane glycoprotein that is highly expressed by human monocytes and tissue macrophages. This protein primarily localises to lysosomes and endosomes with a smaller fraction circulating to the cell surface. It is a type I integral membrane protein with a heavily glycosylated extracellular domain and binds to tissue- and organ-specific lectins or selectins. The protein is also a member of the scavenger receptor family. Scavenger receptors typically function to clear cellular debris, promote phagocytosis, and mediate the recruitment and activation of macrophages'(HGNC:1693).

CD68 is not a macrophage-specific marker (Kunisch *et al.*, 2004): it is expressed in other cells, such as fibroblasts. Different anti-CD68 monoclonal antibodies are commonly used to detect macrophages using IHC. They are KP1, EBM11 and PGM1. Kunisch and colleagues (Kunisch et al., 2004). studied them in terms of specificity of expression by the macrophages and other cells. They found an overlap between CD68 (MoAb KP1 and EBM11) and the fibroblast marker CD90. On the other hand, PGM1 MoAb was less expressed by the synovial lining fibroblasts. Therefore, CD68 (MoAb PGM1) was selected for use in this research to obtain the true representation of the macrophages in the synovial tissue.

2.5.3 Fibroblasts

2.5.3.1 Vimentin

Vimentin is a protein encoded by the *VIM* gene in humans. It is an intermediate filament that is expressed in mesenchymal cells. It is part of the cytoskeleton that maintains the cell structure (Fuchs and Weber, 1994). It is essential for human and animal cells to maintain cell structure to be able to protect the integrity of the cellular components. This has created a challenge to the tissues and resulted in a mechanism to cope with mechanical stress. Human and animal tissues have the capacity to withstand mechanical stress as a result of the presence of the intermediate filaments. The cells in any tissue are not floating in the matrix. Instead, there are cell–cell junctions, such as demosomes, adherens junctions, gap junctions and tight junctions. Along with the intermediate filaments, these junctions create integrated cellular networks that have the stability and flexibility to withstand normal physiological mechanical stresses (Herrmann *et al.*, 2007).

Vimentin is highly expressed by fibroblasts with some expression in T- and B-lymphocytes. It also has an essential role in wound healing as it coordinates fibroblast proliferation and keratinocyte differentiation (Cheng *et al.*, 2016). Ivaska and colleagues (Ivaska et al., 2007) reviewed the novel functions of vimentin and it is interesting to know that it is not exclusively associated with the mechanical and structural properties of the cells. It participates in cell adhesion, migration and invasion, signalling, differentiation, cytoskeletal rearrangements, and regulation of cell morphology and plasticity. One of the important functions related to this research is tissue repair in patients with arthritic joints. Thus, vimentin is expressed more by the fibroblasts in the synovium in cases of tissue repair, especially in arthritis where the damage is continuous. It would be interesting to know how vimentin is related to other inflammatory cells in the synovium as it is essential in cell migration and adhesion. Table 2.1 summarises its functions (Ivaska *et al.*, 2007).

Table 2.1: Summary of vimentin functions in different physiological processes and tentative or identified molecular interaction targets involved in the observed effects *(Ivaska* et al.*, 2007)*

Cellular function	Target of regulation	Effect
Structural integrity of cells and tissues	Endothelial cell junctions and/or ECM interaction	Integrity of cell layers and tissues
Adhesion and migration	Integrins, cell adhesion molecules, cytoskeletal crosslinking proteins	Formation and turnover of adhesive structures
Signal transduction	Transcription factors	Modulation of activity through sequestration
	Protein kinases	Operation and organisation of kinases
	14-3-3	Modulation of 14-3-3 interactions
	Receptors and receptor-associated proteins	Formation of a functional cell surface complex
Apoptotic and immune defence	Cell death-regulating factors	Modulation of activity through sequestration
	Viral components	Regulation of subcellular localisation
	Extracellular domains	Activation of innate immune system cells
Regulation of genomic DNA	Genomic DNA	DNA recombination and repair

2.5.4 B and T cell lymphocytes

2.5.4.1 CD3

CD3 is very well studied and is an integral part of the panel of markers used in the analysis of lymphoid malignancy. It is associated with the human T-cell receptor. The T-cell receptor (TCR) is present in almost 95% of T-lymphocytes as a surface membrane complex. It consists of an α–β heterodimer and at least four monomorphic, invariant T3 or CD3 molecules (γ, δ, ε, ζ). It appears likely that the TCR α–β molecule alone determines both antigen recognition and major

histocompatibility complex (MHC) restriction on at least some T cells. Chemical cross-linking studies of CD3-positive T-lymphocytes that are unreactive with monoclonal antibodies against the TCR α–β molecule revealed that the TCR γ and δ genes are also associated with the CD3 glycoprotein complex. The α, β and CD3 receptor subunits are transmembrane proteins, and all but the ε subunit have *N*-linked glycan moieties (Chetty and Gatter, 1994).

CD3 is a very specific antigen that is strongly expressed in the T-cell lineage. However, there is weak expression in Purkinje cells in the brain, macrophages, and Hodgkin's and Reed–Sternberg cells. It is expressed as a surface membrane in mature T cells. Immunohistochemical studies have shown that CD3 is present in the cytoplasm before expression on the cell surface. 95% of thymocytes express surface membrane and/or cytoplasmic CD3 positivity. The presence of intracellular CD3 has suggested that the cell is going in the direction of the T cell lineage. CD3 helps in the response of T cells to antigenic stimulation through signal transduction. It delivers a biochemical signal from the surface to the inside of the cell after the antigen has bounded to the receptor. CD3 also helps in antigen recognition by the T-cell lymphocytes along with the antigen receptors forming a complex, which are linked non-covalently. CD3 antigen specificity for T cell and its early appearance in T-cell maturation makes it an ideal pan-T-cell marker for T-cell neoplasm. Cabecadas and Isaacson (Cabecadas and Isaacson, 1991) identified three key T-cell antibodies: CD3, UCHL1 and MT1. They assessed their efficacy in histopathological diagnosis of lymphoid and non-lymphoid tissues. UCHL1 (CD45RO) and MT1 (CD43) are not lineage-specific and react with both lymphoid and non-lymphoid cells. MT1 stains T-cell areas specifically in cases of reactive lymphoid tissue. However, MT1 is not lineage-specific as it cross-reacts with granulocytes, megakaryocytes, brain cells and cases of high- and low-grade. B-cell UCHL1 is also non-lineage specific because it cross-reacts with similar tissues to MT1. Cabecadas and Isaacson found that both UCHL1 and MT1 have reactivity with a high percentage of T-cell lymphomas (94 and 86%, respectively), but lacked absolute specificity for T cells, especially in high-grade lymphomas. CD3 was the most specific of the three T-cell markers, but stained only 78.5% of cases in their study. In all of the CD3-negative cases, except for one

anaplastic large cell lymphoma of the skin, UCHL1 was also positive. This suggests that a panel of T-cell markers including the key reagents UCHL1 and CD3 should be used as part of the routine diagnostic practice for any lymphoid neoplasm.

In our research, which is non-diagnostic, CD3 alone was used as a T-cell marker to find the expression in the synovial tissue.

2.5.4.2 CD20

A lot of attention has focused on lymphocyte development and function in the last 50 years, which has led to a better understanding of their function and how to create therapeutic agents that could cure diseases like lymphomas. The basic function of B and T lymphocytes is antibody production and cell-mediated immune response. It is interesting to know that the discovery of a B cell originated after identification of a protein (Ig or antibody). It is the recognition of these proteins that led to the discovery of the antibody-producing cell (Tiselius and Kabat, 1938).

The molecular architecture of the B-cell surface was unknown until approximately 1980s and was known to consist of membrane-bound Ig, complement component receptors and Fc receptors. The understanding of the B-cell structure evolved following the advent of monoclonal antibody technology.

B cells are important in the adaptive immune system. In addition to the production of antibodies, they regulate other functions to maintain immune system homeostasis. They produce immunomodulatory cytokines, which have effects on other inflammatory cells like T-cells, DCs, and antigen-presenting cells, regulate lymphoid tissue organisation and neogenesis, regulate wound healing and transplanted tissue rejection, and influence tumour development and tumour immunity. Moreover, B cells are required to initiate the T-cell immune response (LeBien and Tedder, 2008).

The first specific surface antigen to be categorised was B1, now known as CD20 (Stashenko *et al.*, 1980). Tedder *et al.* (Tedder et al., 1988) analysed the B1 (CD20) molecule and noted that it is a phosphoprotein on the surface of human B-lymphocytes. They also found that a predominant species of Mr 33,000 represents

75–80% of the iodinated cell surface B1 and a Mr 35,000 species represents 20–25%. CD20 is a general B-cell marker that is expressed during B-cell differentiation from the pro-B cell phase until the plasma cell stadium (Boross and Leusen, 2012). It is an activated-glycosylated phosphoprotein expressed on the surface of all B cells beginning at the pro-B phase and progressively increasing in concentration until maturity. In humans, CD20 is encoded by the *MS4A1* gene. This gene encodes a member of the membrane-spanning 4A gene family, which encodes a B-lymphocyte surface molecule, which plays a role in the development and differentiation of B cells into plasma cells. The exact function of CD20 is still unknown. However, it is believed to have a role in B-cell activation, regulation of B-cell growth and transmembrane calcium flux (Riley and Sliwkowski, 2000).

CD20 was first used as a therapeutic target in the management of lymphomas. CD20 monoclonal antibody (MoAb) binds to the human CD20 antigen, inducing CD20+ B-cell depletion. Rituximab was the first anti-CD20 MoAb to be approved by FDA for the management of lymphoma. Once bound to the CD20+ cells, MoAb causes cell depletion through three main killing pathways: complement-dependent cytotoxicity, antibody-dependent cell-mediated cytotoxicity and direct pro-tumour activity (Avivi *et al.*, 2013). One of the B-cell functions is antibody production, which is important to control in autoimmune disorders like rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE). Therefore, rituximab is used to control autoantibody production by B-cell depletion and hence control the autoimmunity problem.

This marker was included in this study to provide a complete assessment of the synovium in this group of patients and to see if they show any signs that suggest similarities to RA.

2.6 Rationale of the study

It is still not clear why some people develop OA very late in their life while others get it early. The literature review suggests synovitis and inflammation as major contributors to OA. Increased fat in the synovial membrane will promote chronic

inflammation, which leads to neo-angiogenesis to maintain the metabolic demands of the adipocytes. This, in turn, will lead to more inflammatory cell infiltration and increased inflammation, which is accompanied by damage of the surrounding tissues, leading to early arthritis. Moreover, the synovium of obese people has not been studied properly before. This laboratory research will focus on comparing obese patients' synovium to that of lean people of different age groups and BMIs. There are several questions that are of interest to musculoskeletal scientists when it comes to OA. This research will focus mainly on the angiogenesis and microcirculation of the synovium based on age and BMI.

It is a complex study in terms of population differences and heterogeneity. OA itself makes it a challenging project owing to the multiple risk factors. This study has several limiting factors that could affect the results and interpretation. They will be discussed in the study limitations section (section 5.6).

2.7 The proposed study

All patients with advanced primary OA who have been assessed by clinicians and were eligible for primary knee arthroplasty will be categorised according to their age and BMI into three groups as follows:

- Group 1: The young (<60) and obese (BMI > 30),
- Group 2: The young (<60) and lean (BMI < 30)
- Group 3: The old (>70) and lean (BMI < 30)

These groups have been chosen to see differences among different age groups in relation to their BMI. We understand that we are not comparing like with like and the groups are completely different. This was discussed prior to commencing this project. The synovium of obese people has not been studied properly before. To help balance out the big differences between the old lean and young obese groups, a third group was recruited that shares some common features. This group was young and lean.

One of the points of interest to us is, would there be a significant difference that suggests the early OA in obese patients compared to the old lean patients who present OA late. The lean young group was added to see why this particular group gets early OA despite the absence of obesity as a risk factor. This group will also help to avoid any incorrect interpretation.

The synovium was studied by looking at the histology slides after staining with Hematoxylin and Eosin (H&E) and IHC. The IHC was carried out to see the cells of interest, including endothelial cells, macrophages, fibroblasts, and B and T cells.

- The histology slides were studied for:
    1- The dimensions of adipocytes (H&E only).
    2- Angiogenesis and its relation to the adipocyte size and site.
    3- Presence or absence of synovitis and scoring the synovitis, if present.

- Immunohistochemistry staining was used to identify and count endothelial cells, fibroblasts, lymphocytes and macrophages using the following phenotypic markers:
    – Endothelial: vWF, CD34
    – Macrophages: CD68
    – Fibroblasts: vimentin
    – Lymphocytes: CD3, CD20

2.8 Aims

2.8.1 Primary aim

To study the effect of obesity on synovial tissue as a source of chronic inflammation, angiogenesis and synovitis leading to the development of OA.

2.8.2 Secondary aims

1- To study the effect of fat in the synovial tissue and it relation to angiogenesis.

2- To assess the changes in microcirculation in the synovium.

3- To explore the relation between vascular changes and other inflammatory cells.

4- To determine the predominant inflammatory cell type in all the three groups.

5- To assess the synovitis in all the groups.

3. MATERIALS AND METHODS

3.1 Hypotheses

Vascular and inflammatory components of synovial tissue, debrided at primary knee arthroplasty for OA, will vary with age and BMI. The increased fat leads to neo-angiogenesis and hence inflammation, which causes the early onset of OA.

3.2 Null hypothesis

The hypotheses are individually or collectively not true

3.3 Study population recruitment

3.3.1 Study participants

All patients presenting to Royal Liverpool and Broadgreen University Hospital NHS trust with established primary knee osteoarthritis who were indicated for total knee arthroplasty were eligible for the study.

3.3.2 Inclusion criteria

- Patient was willing and able to give informed consent for participation in the study.

- Age > 70 with BMI < 30.

- Age < 60 with BMI > 30.

- Age < 60 with BMI < 30.

- Diagnosed with idiopathic OA.

- Undergoing primary knee arthroplasty for advanced end-stage OA.

3.3.3 Exclusion criteria

Patients were excluded from the study if ANY of the following applied:

- Evidence of lower limb gangrene/ulcers or peripheral vascular disease.

- History of hepatic or renal impairment or dialysis.

- Currently receiving or has received radiation or chemotherapy within the last 3 months.

- Had inadequate venous access for drawing blood.

- Prior or current use of systemic cortisone.

- Any other significant disease or disorder that, in the opinion of the investigator, might either put the participants at risk because of participation in the study, or influence the result of the study or the patient's ability to participate in the study.

- Lack the capacity to give consent.

- Secondary OA, such as inflammatory or post traumatic arthritis.

3.3.4 The number of participants

This study focuses on angiogenesis in relation to the fat cells in the synovium in patients with primary OA in different age groups and BMIs. The Von Willibrand Factor (vWF) is the main marker of interest to detect new endothelial cell formation. The literature shows that vWF is a very sensitive marker in detecting angiogenesis (Pessler *et al.*, 2008a, Pessler *et al.*, 2008b, Mucke *et al.*, 2016). The process of releasing the vWF during angiogenesis has been studied by (Babich et al., 2008). Other factors of interest in this study are CD3, CD20, CD34, CD68 and vimentin.

G*Power software was used to estimate the sample size using the previous findings of (Pessler et al., 2008b). This paper showed that the mean vWF level in normal individuals was 61.5 with a standard error of the mean (SEM) of 3.7, compared with 85.9 (5.9) and 85.4 (7.2) for orthopaedic arthropathies and osteoarthritis, respectively. The standard deviation (SD) of the control group was

calculated from the data available (SD = 11.7). The details of Pessler's statistical analysis are shown in Table 3.1. RA was excluded from the analysis as it is not in the scope of this study. In this project, we are examining three different groups, which are most likely not normally distributed. Thus, an ANOVA fixed effect one way test was used. The data was plotted in G*Power using the ANOVA fixed effect one way test to determine the sample size and the power of the study.

With a power of 85% and alpha error of 0.05, five patients are required in each group to produce a statistical significance for the vWF level. The same test was run to assess the power when using five patients in each group for the other factors of interest (CD20, CD3, CD68). CD20 and CD68 will be well powered (over 99%) when using five patients. On the other hand, CD3 would be underpowered.

Table 3.1: Data of synovial markers from Pessler et al.

Assessment	Normal	Orth. A	OA	RA
Lining thickness†	1.31 (0.05)*	1.78 (0.13)	1.62 (0.12)	4.86 (0.62)*
Synovitis score‡	1.38 (0.28)	2.07 (0.24)	2.23 (0.29)	5.74 (0.33)*
CD3††	27.7 (9.0)	51.1 (11.0)	67.2 (11.7)	552.9 (80.0)*
CD20††	2.3 (1.2)	32.3 (21.1)	16.5 (6.6)	251.4 (47.4)*
CD38††	0.3 (0.2)*	15.4 (8.1)	23.3 (10)	625.5 (95.8)*
CD68				
Lining, total	8.2 (1.9)	11.1 (1.6)	19.3 (2.2)*	26.9 (1.9)*
Lining %§	36.1 (6.2)	38.3 (5.5)	57.2 (6.1)*	72.7 (4.4)*
Subintima††	45.6 (7.4)	93.1 (18.3)	154.8 (18.8)*	579 (50.)1*
Total inflammatory cells¶	75.9 (18.3)	192.0 (44.3)	261.8 (31.9)*	2008.8 (203)*
IHC inflammation score	0.19 (0.06)*	0.74 (0.23)	1.26 (0.26)	23.2 (2.3)*
Ki-67‡‡	10.2 (3.3)	12.5 (3.9)	31.1 (5.2)*	196.6 (33.2)*
vWF+ vessels**	61.5 (3.7)*	85.9 (5.9)	85.4 (7.2)	118.4 (8.9)*

Results are shown as mean (SEM).

3.3.5 Participant recruitment to the LMB

A member of the local clinical or research team within the LMB identified participants with established knee arthritis presenting to the outpatient trauma/orthopaedic clinic or on admission for surgery. The eligibility criteria were checked and those patients potentially suitable for inclusion were given information about the LMB and invited to discuss the role of the LMB further with a member of

the research team. Those who were willing to participate were given the opportunity to ask questions and the appropriate informed consent was obtained. All routine patient care followed good clinical practice guidelines. Once consent was signed, participants were recruited in the LMB and baseline outcome data were collected in the outpatient clinic.

3.3.6 Sample collection

For those patients undergoing knee arthroplasty, tissues were collected as per the LMB protocol. The surgeon who performed the procedure took the samples from the suprapatellar pouch and medial and lateral gutters. The samples were then sent to the LMB for storage as explained in the sample handing section. The stored synovium was accessed through the LMB for analysis according to the study protocol. Tissue samples were used for histology and immunohistochemistry.

3.3.7 Sample handling

Tissue samples were placed in containers containing RNAlater or formalin and were prepared for dispatch, including anonymising the sample using the participant's unique study number, initials, date and time. A good clinical practice (GCP)-trained research nurse or fellow transported the samples to the LMB. Samples were divided into adequate sizes and allocated for the study investigations. Only those involved in the research process had access to the samples.

3.3.8 Blinded assessors procedure

Samples were link-anonymised and blinded using the participant's unique study number. The assessor of the samples in this case had to know the details of the patients to make sure they satisfied the inclusion and exclusion criteria. Information on patient age and BMI were essential to allocate the patient to the correct group and to make sure that the number of participants in each group was similar. Blinding

was done at the time of assessment of the IHC slides. This was to ensure assessor blinding to reduce bias. However, there were situations where the assessment was not blinded to look at hot spots (areas with severe inflammation or synovitis) in the whole slide, which was difficult to blind.

3.3.9 Discontinuation/withdrawal of participants

Each participant had the right to withdraw from the study at any time. The research team would record any reason for any withdrawal on the study Withdrawal CRF and the participant would be asked if the study team might use the data collected up to the point of withdrawal.

3.3.10 Ethics approval

Ethical approval was obtained by the chief investigator for sample collection to go to the Liverpool Musculoskeletal Biobank (REC reference number 15/NW/0661 dated 9th September 2015). The study was carried out under the supervision of the research team outlined above after obtaining sponsorship from the University of Liverpool.

3.3.11 Role of Liverpool Musculoskeletal Biobank (LMB)

LMB is an HTA (Human Tissue Authority)-approved research biobank under ethical approval of the central Liverpool ethics committee (reference 15/NW/0661). The LMB sponsor is the University of Liverpool (reference UOL001150) operating under HTA license reference 12020. The LMB staff are responsible for obtaining consent from participants, collection of tissues and data, samples logging, sample tracking, and storage and disposal of samples as per LMB-approved standard operating procedures. The samples were accessed through the LMB after obtaining

sponsorship from the University of Liverpool and getting approval to start recruiting the patients (reference: UOL001283).

3.4 The Laboratory work

The groups were allocated as follows (Figure 3.1):

Group 1: Young obese (six patients)

Group 2: Young lean (five patients)

Group 3: Old lean (six patients)
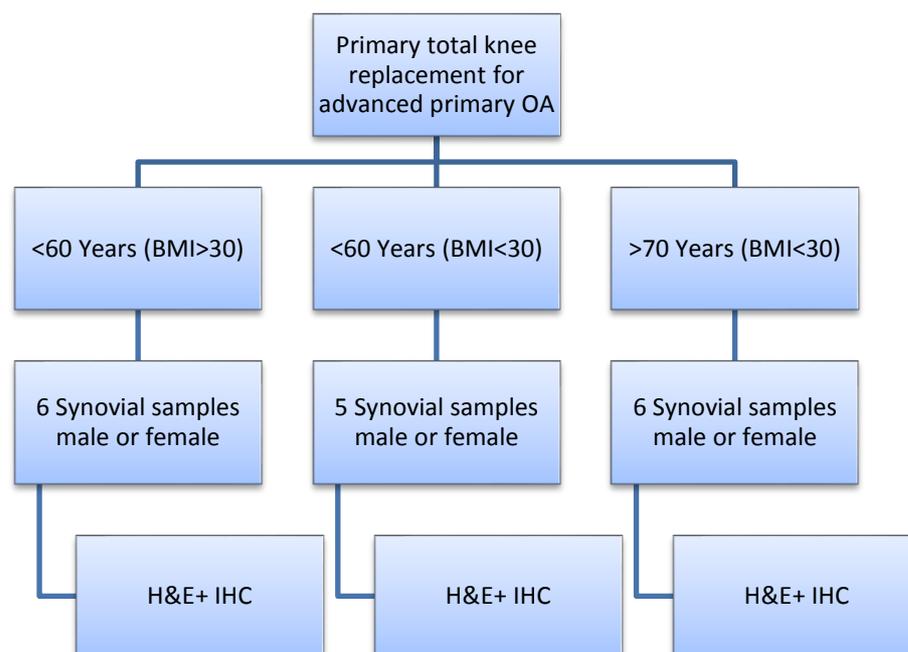
Figure 3. 1 : *Study population (see Appendix 1 for the study flowchart).*

The tissue samples were collected from the LMB stored in paraffin blocks. The patient samples were allocated to the specific groups based on age and BMI after satisfying the inclusion and exclusion criteria. The paraffin blocks were amalgamated in such a way that each group was in one composite paraffin block. This idea was to

reduce the time and cost involved in block sectioning and staining. It also helped reduce the number of slides for each marker, which helped during IHC staining to apply the same treatment across all the groups without any discrepancy in time. After the power calculation, the total number of patients recruited was 17. Tonsil tissue was used as a positive control and was placed in the group 2 composite block. The block designs are shown in Figure 3.2.

Group 1 (Young obese)	Patient 038	Patient 020	
		Patient 068	
	Patient 080	Patient 050	Patient 057

Group 2 (Young lean)	Patient 028	Patient 071	
		Patient 070	
	Patient 069	Tonsil tissue	Patient 041

Group 3 (Old lean)	Patient 023	Patient 072	
		Patient 030	
	Patient 019	Patient 033	Patient 052

Figure 3. 2: Map of patient location in the composite block and the histology slides in all the groups.

The patient tissues were arranged in the composite block in this irregular way to avoid mixing the samples up when sectioning and fixing the tissue to the slides. This was to allow the investigator to know which patient is at which position based on the map provided in Figure 3.2.

After sectioning the blocks, H&E staining was done followed by the IHC staining for the following markers: CD3, CD20, CD34, CD68, vWF and vimentin. Table 3.2 details the type of antibody and titration.

Table 3.2: Antibodies used for immunohistochemistry.

Primary antibody	Source	Isotype	Clone	Dilution	Treatment	Linker
vWF	DAKO		Polyclonal rabbit	1:1000	High PH	Rabbit linker
CD 34	DAKO	IgG1	Monoclonal mouse (class 2 QBEnd 10)	1:50	High PH	Mouse linker
CD 3	DAKO	IgG3	Monoclonal mouse	Ready to use	High PH	None
CD 20	DAKO	IgG3	Monoclonal mouse	Ready to use	High PH	None
CD 68	DAKO	IgG3	Monoclonal mouse (PG-M1)	1:600	High PH	None
Vimentin	DAKO	IgG2a	Monoclonal mouse (V9)	1:200	High PH	None

The H&E staining was done by the Liverpool Bio-Innovation Hub Biobank, where they use an automated H&E staining machine (Leica Autostainer).

The IHC staining was carried out using the autostainer as follows:

- Envision-Flex block was incubated at room temperature for 5 minutes.
- Primary antibody incubated for 30 minutes after dilution to appropriate concentration using antibody diluent (Envision-Flex).
- Appropriate linker as outlined in Table 1.2 (*i.e.* mouse for

mouse primary antibody) was added and incubated at room temperature for 15 minutes.

- HRP was added and incubated at room temperature for 20 minutes.

- DAB added and incubated at room temperature for 20 minutes.

- The slides were unloaded from the autostainer and transferred into a dH$_2$O bath.

- Counterstaining with haematoxylin solution was done for 1 min.

- The slides were then dipped in acidic alcohol and rinsed in tap water

- Next, the slides were dipped in ammonia water for 30 sec and rinsed in tap water before returning to the bath of dH$_2$O.

- The slides were dehydrated through five changes of industrial methylated spirits and two changes of xylene in a fume hood.

- Finally, the slides were mounted in DPX.

These markers had already been worked up in the Royal Liverpool and Broadgreen University Hospital NHS Trust laboratory and similar markers were used in this project with the same dilution as outlined in the table 3.2.

An autostainer was used to insure that all the slides received similar treatment and to avoid any potential human error. The positive control was a tonsil tissue. The negative control was the patients' own tissues but without the antibody treatment. This was done to eliminate any non-specific staining that could affect the results. The positive control showed good uptake of all the markers. The negative control did not show any non-specific uptake. A trained person supervised the staining procedure to ensure that all the steps were carried out correctly. The details of the staining procedure are shown in Appendix 2.

The slides were examined for staining quality by the supervisor. The staining was satisfactory and there was no need to repeat any markers. Subsequently, the slides were scanned using an **APERIO IMAGE SCANNER** and the slides were ready to be analysed. The images were opened using **ImageScope** software. Multiple images were taken at ×10 magnification to cover the whole slide for each marker. The images were divided into either synovial or fat based on the major tissue. The images were labelled as synovium if >50% of the specimen was synovial tissue with cells and stroma and fat if >50% of the tissue was fat. So, each slide would have fatty and synovial sections.

3.4.1 Immunohistochemistry

Immunohistochemistry (IHC) is a powerful method for localising specific antigens in formalin-fixed, paraffin-embedded (FFPE) tissues based on antigen–antibody interactions (Taylor and Burns, 1974). It relies on antigen–antibody interactions to identify cellular or tissue constituents. The site of antibody binding is identified by direct labelling of the antibody, or by use of a secondary labelling method (Bancroft JD, 2008). IHC is a well-established technique (Figure 2.2).
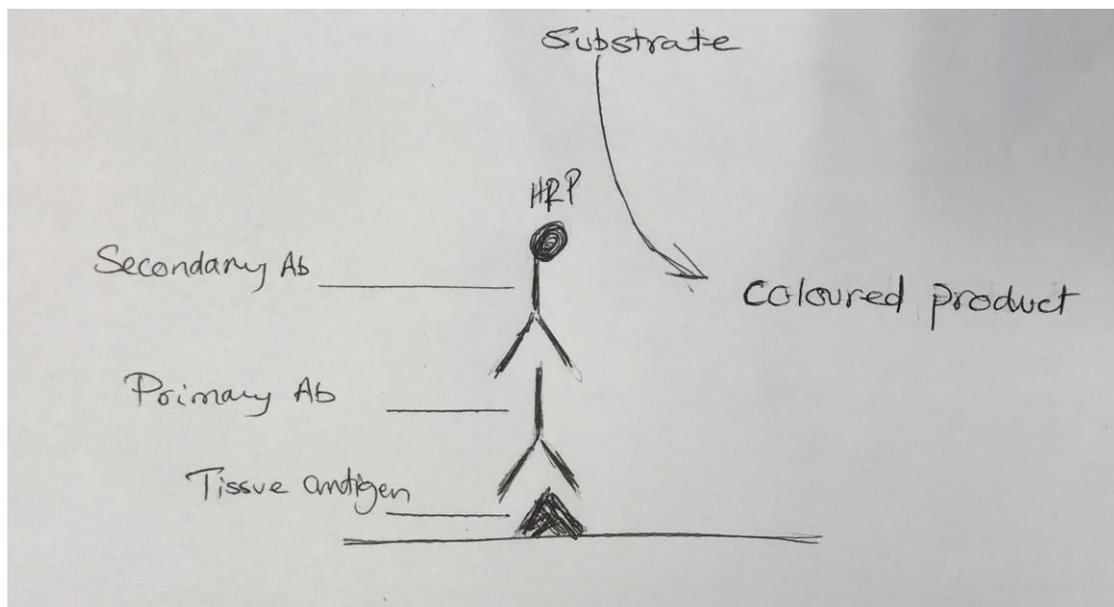
Figure 2. 3: diagram showing how IHC works (adapted from Prestige Antibodies)

There are essential steps that must be carried out before staining, including tissue fixation. It is important to preserve the tissue from changes that may affect

the cellular and extracellular components. Formalin is widely used for this purpose. Although formalin is very good at fixing the tissue, it has some unwanted effects like crosslinking between proteins themselves and proteins with nucleic acids. It also can mask or damage epitopes through alteration of the protein's dimensional structure. The fixed tissues then go through a process of embedding in paraffin blocks. This process involves alcohol treatment followed by xylene, and then embedding in the paraffin block before storage.

IHC involves the following steps:

3.4.1.1 Sectioning

The FFPE tissue is sectioned into slices of 4–5 µm thickness and mounted on a slide.

3.4.1.2 Antigen retrieval

The paraffin must be completely removed from the FFPE sections for the antibodies to reach the target antigens (antigen retrieval). There are different antigen retrieval methods available. Heat-induced epitope retrieval using the Dako PT-Link with high pH was used in this project. The protocol for PT-Link antigen retrieval is included in the appendix 2.

3.4.1.3 Blocking endogenous target activity

Peroxidases, most often horseradish peroxidase (HRP), are commonly used as the reporter for target antigen detection by IHC. Final analysis of this staining method is complicated by the presence of endogenous peroxidase and "pseudoperoxidase" activity in the cells and tissues. Endogenous peroxidase reacts with hydrogen peroxide to reduce the 3,3'-diaminobenzidine (DAB) substrate or other peroxidase substrates, resulting in nonspecific staining of the tissue. To prevent false positive and high background detection of the stain, blocking of endogenous forms of peroxidases or phosphatases is important. A commercially available chemical blocking agent was used (Dako Envision-Flex block, Dako UK Ltd.).

3.4.1.4 Immunodetection

IHC target antigens are detected through either chromogenic or fluorescent means. Chromogenic detection is based on the activities of enzymes, most often HRP or alkaline phosphatase, which form coloured, insoluble precipitates upon the addition of the substrate, such as DAB and nitro blue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP), respectively. HRP (Dako UK Ltd) was used for this step.

3.4.1.5 Sealing the stained sample

Once the staining procedure is done, the slide is sealed by mounting a coverslip with an appropriate mountant to stabilise the tissue sample and stain. This allows preservation of the sample for long-term use and storage.

3.4.1.6 Controls

Controls are used to validate IHC results.

a) Positive control

This is a tissue that is known to contain the antigen of interest detected by identical IHC methods to those used in diagnostic cases. Positive tissue controls must be fixed and stained in the same way as the diagnostic case tissue for every antibody and procedure used (José A. Ramos-Vara, 2008). Tonsil tissue was used as the positive control in this work.

b) Negative controls

The same tissue can be used but the primary antibody is omitted from the staining schedule. It is replaced by an immunoglobulin, which is directed against an unrelated antigen. Commonly, the primary antibody is replaced by antibody diluent, same species non-immune immunoglobulin of the same dilution and immunoglobulin concentration, an irrelevant antibody or buffer. These methods will assess the degree of cross-reactivity of the primary antibody and the degree of nonspecific binding by the labelling (secondary) antibody and detection system. In

this project, we used the patient's tissue samples as a negative control to detect any non-specific staining.

The IHC staining protocol that was used in this was adapted from the Liverpool Ocular Oncology Research Group (Appendix 2).

3.4.2 IHC analysis and assessment

Image analysis was done using ImageJ software (version Fiji-win64). The markers were quantified as percentage of stain following Dr. Ashish N. DebRoy (N, 2012) technique using ImageJ software (N, 2012). The same technique was also used by Mucke (Mucke et al., 2016) and colleagues for assessment of inflammation in the synovium (Mucke *et al.*, 2016). The technique relies on choosing the colour deconvolution that represents DAB, then the threshold is readjusted for every marker separately to get rid of the background noise. After that, H DAB vector is used to isolate the DAB stain. The software then measures the total DAB percentage, which is related to the specific tissue, based on the antibody used.

3.4.2.1 Blinding

The images were blinded by the supervisor using the ImageJ software to ensure that bias was eliminated while doing the assessment. Each marker for all the groups was saved in a separate folder to allow access and specific threshold while using ImageJ. The author was blinded and did not know which image was related to which patient until all the measurements were taken. After the completion of the ImageJ quantitative measurements, the blinding was removed. Blinding was done for the ImageJ analysis only.

3.4.2.2 Quantitative measurements using ImageJ software

ImageJ software was used to quantify the percentage of stained cells in the whole slide. A threshold was measured for each stain to detect the DAB marker. It relies on the percentage of the stained cells in a given area. The DAB percentage was

measured in the whole slide for each marker. Each marker was assessed with a specific threshold to avoid counting any background noise in the image. The DAB percentage of each image was recorded. Both the synovial and fatty sections were analyzed.

3.4.3 Other manual measurements

3.4.3.1 Vascular density in the intimal layer of the synovium

Vascular density was used to count the vessels in the hot spots. A hot spot was defined as the area with the highest number of vessels and inflammatory cell infiltration in the intimal layer of the synovium. Only fields with clearly recognizable intimal and subintimal vasculature were included (Pessler *et al.*, 2008a). This calculation was done at ×10 magnification using the vWF as a marker of endothelial cells. The number of vessels in the power field was counted (Pessler *et al.*, 2008a). The vascular density was further divided into vessels that had a smooth muscle layer and those that did not have any smooth muscle layer. The vessels that had a smooth muscle layer represented the arterioles whereas those that did not have a smooth muscle layer represented the capillaries.

3.4.3.2 Vascular density in the fat sections

The same technique of vascular density measurement was done in the fat sections. The areas were chosen in places where only fat cells were present and the sections were not distorted. Only the number of vessels present in the power field was counted. There was no further division of vessels with or without smooth muscle layer.

3.4.3.3 Vascular wall thickness

The slides were examined for vascular hyperplasia. The worst section was chosen from each slide at ×10 magnification where a collection of vessels with vascular wall

hyperplasia was present. These vessels were chosen to be very close to the synovial intima to avoid deeper sections were the vessel can be bigger in size normally. The mean vascular wall thickness was measured in these areas. The thickness was measured from the inner surface of the vascular lumen (tunica intima) to the outer surface of the tunica media, which is also known as the intima–media thickness (IMT). IMT is an indicator of subclinical atherosclerosis that gives an assessment of cardiovascular disease risk and is usually measured in the carotid by ultrasound scan (Stein *et al.*, 2008, Han *et al.*, 2016). This was examined in vessels very close to the synovial intimal and sub-intimal layers. The slide was not included if there was no representative section of the synovium.

3.4.3.4 Vessel distance from the joint-facing synovial surface

The distance of the nearest vessel to the joint-facing synovial surface was measured in the same palaces where the vascular density was measured. The measurement was taken at magnification of ×10. The average was taken for the distance (Levick, 1995).

3.4.3.5 Fat cell diameter

To assess the fat cell size, ideally adipocytes should be isolated by collagenase digestion (Fang *et al.*, 2015). However, in this project we relied on H&E staining for assessment of fat cell size. This measurement was used to assess the local fat burden as a tool of the local effect of obesity. The diameters of the fat cells were measured from different places in each slide where the fat cells were intact and the wall was clear with no signs of disruption of the tissue. This was done manually in micrometres using the image scope software measurement tools. The H&E-stained slides were used for this measurement. The average was calculated and plotted on the data sheet. The measurement followed (Fernandes-Santos et al., 2009) fat cell diameter method.

3.4.4 Semi-quantitative assessment

Semi-quantitative assessment of the number of the stained cells was done following Dr Parashar and Professor Helliwell's (Parashar; and Helliwell, 2012) method of assessment on the synovium(Parashar; and Helliwell, 2012). Cell expression was categorised based on the number of stained cells in the histology slide. It was classified as no expression (0), few positive cells (1+) and many positive cells (2+). The positive and negative tissue samples were used as a baseline from which to determine the strength of the expression. Table 3.3 shows the simple description of the semi-quantitative assessment.

Table 3.3: Description of semi-quantitative scoring system

Score	Description
0	No expression
1+	Few positive cells
2+	Many positive cells

3.4.5 Chronic synovitis scoring

The H&E-stained histology slides were evaluated for the presence or absence of chronic synovitis using Krenn *et al*.'s (Krenn et al., 2006)scoring system (Krenn et al., 2006). Table 3.4 outlines the components of the scoring system

Table 3.4: Chronic synovitis scoring system (Krenn *et al.*, 2006).

Enlargement of the synovial lining cell layer	
0 points	The lining cells form one layer.
1 point	The lining cells form 2–3 layers.
2 points	The lining cells form 4–5 layers, few multinucleated cells might occur.
3 points	The lining cells form more than 5 layers, the lining might be ulcerated and multinucleated cells might occur.
Density of the resident cells	
1 point	The cellularity is slightly increased.
2 points	The cellularity is moderately increased, multinucleated cells might occur.
3 points	The cellularity is greatly increased, multinucleated giant cells, pannus formation and rheumatoid granulomas might occur.
Inflammatory infiltrate	
0 points	No inflammatory infiltrate.
2 point	Numerous lymphocytes or plasma cells, sometimes forming follicle-like aggregates.
3 points	Dense band-like inflammatory infiltrate or numerous large follicle-like aggregates.
Sum 0–1	**No synovitis**
Sum 2–4	**Low-grade synovitis**
Sum 5–9	**High-grade synovitis**

3.5 Statistics and analysis

Variables collected were subjected to normality test using the Shapiro–Wilk test. Parametric data were described as mean ± standard deviation whilst non-parametric data were reported as median with ranges. With regards to categorical data, they were explained in percentages.

The sample size is small and the data is non parametric. Thus, for comparison of more than groups the Kruskal–Wallis test was used and the Mann–Whitney U test was used for two groups. The Spearman test was used to assess correlations between variables.

4. RESULTS

4.1 Demographics

4.1.1 Age and BMI

A total of 17 patients were recruited to this study and divided into three groups. Group 1 represented the young obese patients. Six patients were allocated to group 1 with a mean (SD) age of 53.3 (2.33) and BMI of 38.53 (1.16). Group 2 had five patients with a mean (SD) age of 55.60 (2.33) and BMI of 27.01 (1.59), and six patients were allocated to group 3 with a mean (SD) age of 75.33 (3.2) and a BMI of 25.64 (2.5).

The Shapiro–Wilk test was used to assess the normality of the age and BMI data and it was not normally distributed. Across-groups comparison was done using the Kruskal–Wallis test and the data showed a significant difference across the groups for both age and BMI. There was no significant difference in the ages between groups 1 and 2. Group 3 was significantly different when compared to group 1 (P = 0.02). On the other hand, BMI was significantly different between groups 1 and 2 as well as groups 1 and 3, with P values of 0.043 and 0.004, respectively. This shows that groups 1 and 2 are matched for age (<60) whereas groups 2 and 3 are matched for BMI (<30). Table 4.1 shows the details of the statistics.

Table 4.1: Group distribution according to age and BMI

	Groups (N = 17)			P value
	Young Obese (G1, n = 6) (Age < 60, BMI > 30)	Young Lean (G2, n = 5) (Age < 60, BMI < 30)	Old Lean (G3, n = 6) (Age >70, BMI < 30)	
	Mean Median Range SD	Mean Median Range SD	Mean Median Range SD	
Age	53.3 54.00 (49–55) 2.33	55.60 58.00 (48–59) 4.50	75.33 74.50 (71–80) 3.2	**Across all groups : p= 0.02 [a]** G1 = G2 : p =1.00 [b] **G1 < G3 : p = 0.02** [b] **G2 < G3 : p = 0.063** [b]
BMI	38.53 38.41 (36.8–40.2) 1.16	27.01 26.74 (25.7–29.3) 1.59	25.64 25.39 (22.4–40.3) 2.5	**Across all groups : p= 0.03 [a]** **G1 > G2 : p =0.043** [b] **G1 > G3 : p = 0.004** [b] G2 < G3 : p = 1.00 [b]

Note. a = Kruskall–Wallis test.   b = Dunn–Bonferroni *post hoc* test.

Figures 4.1 and 4.2 present the medians of each group and show how they are distributed.
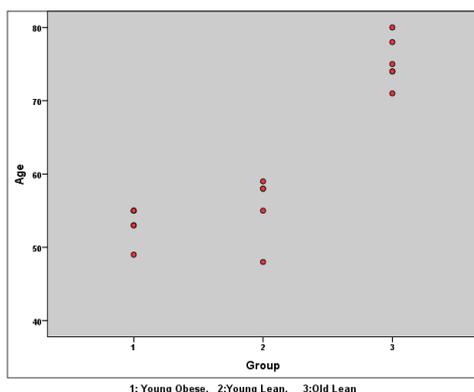
Figure 4. 1: Age distribution
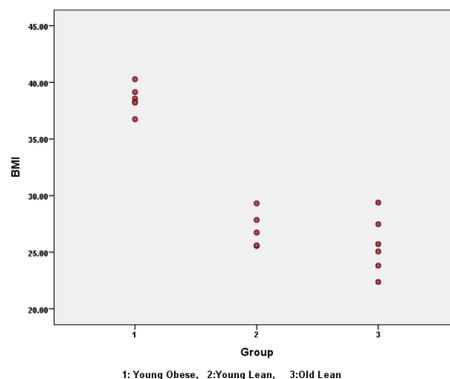
Figure 4. 2: BMI distribution

4.1.2 Other clinical data

Other parameters were also obtained through the LMB database. The data obtained included: gender, smoking status, ASA grade, comorbidities, Kellgren and Lawrence (KL) score, metabolic syndrome, use of statins and non-steroidal anti-inflammatory drugs (NSAIDs), number of painful joints, WOMAC score and SF12 score. As shown in Table 4.2, the male to female ratio is almost similar in group 1 and 2 but in group 3 there is more female than male representation (F:M = 2:1).

There were more smokers in group 2 than in the rest of the groups. The project population showed good general health and the overwhelming majority were ASA grade 2. All three groups had few comorbidities. Approximately half of group 1 and 2 suffered with one comorbidity. On the other hand, the majority of group 3 patients were healthy. No patients fulfilled the criteria of metabolic syndrome. In addition, none of the patients were using lipid-lowering medications (statins). A few patients from group 1 and 2 were using NSAIDs. However, all the patients in all the groups shared the same advanced OA score (KL score). The average number of painful joints in each group was three. They did not show any significant differences in their functional scores including WOMAC and SF12. Although smoking is more common in group 2, comparison of the smokers and non-smokers did not show any significant differences in the distribution of the IHC markers when using the Mann–Whitney U

test.

Table 4.2: Other clinical parameters (*= see appendix 4, **= see appendix 3)

	Groups (N=17)		
	Young Obese (G1, =6) (Age<60, BMI>30)	Young Lean (G2, n = 5) (Age<60, BMI<30)	Old Lean (G3, n = 6) (Age>70, BMI<30)
Sex			
M	3	2	2
F	3	3	4
Smoking			
Yes	1	4	1
No	5	1	5
ASA			
1	0	1	0
2	5	3	6
3	1	0	0
4	0	0	0
5	0	0	0
Comorbidities			
Yes	3 (1 in each affected patient)	3 (1 in each affected patient)	2 (1 in each affected patient)
No	3	2	4
KL score			
1	0	0	0
2	0	0	0
3	0	0	0
4	**6**	**6**	**6**
MetS			
Yes	0	N/A	N/A
No	6		
Statins			
Yes	**0**	**0**	**0**
No	6	5	6
NSAIDs			
Yes	2	1	0
No	4	4	6
No. of painful joints (average)	3	3	3
WOMAC score* (average)	81	68	67
SF 12 (average)**			
PCS	22.35	31.99	29.54
MCS	49.45	54.932	50.91

4.1.3 Summary of demographic data

The project population was chosen carefully to avoid any factors that could affect the results. They are almost similar except for the age and BMI. They are all at the same stage of advanced knee arthritis with no significant difference in their functional scoring. The

comorbidities were evenly distributed and this was reflected in the ASA score. Although group 2 had more smokers compared to the other groups, no significant differences were observed in the distribution of markers among the smokers and non-smokers. Of course, the sample size is too small to confirm the effect of smoking on the markers.

4.2 H&E stain analysis

The synovial samples were heterogeneous with the sections varying in size and tissue components. Some samples showed pure cellular and stromal components whilst others showed a mixture of fatty and cellular components. The ratio of the pure synovial cellular sections to the fatty sections was also variable. The fat representation was variable among patients. There were also differences in the synovitis and inflammatory infiltrations. The fat cells in some sections were distorted. All these differences were assessed systematically in the following sections. Figure 4.3 shows examples of H&E-stained sections taken at ×5 magnification for patients from different groups.

There were some interesting findings in the morphology of the microvasculature of the synovium where vascular hyperplasia was observed in the synovial sections. They were more pronounced in the young obese people.
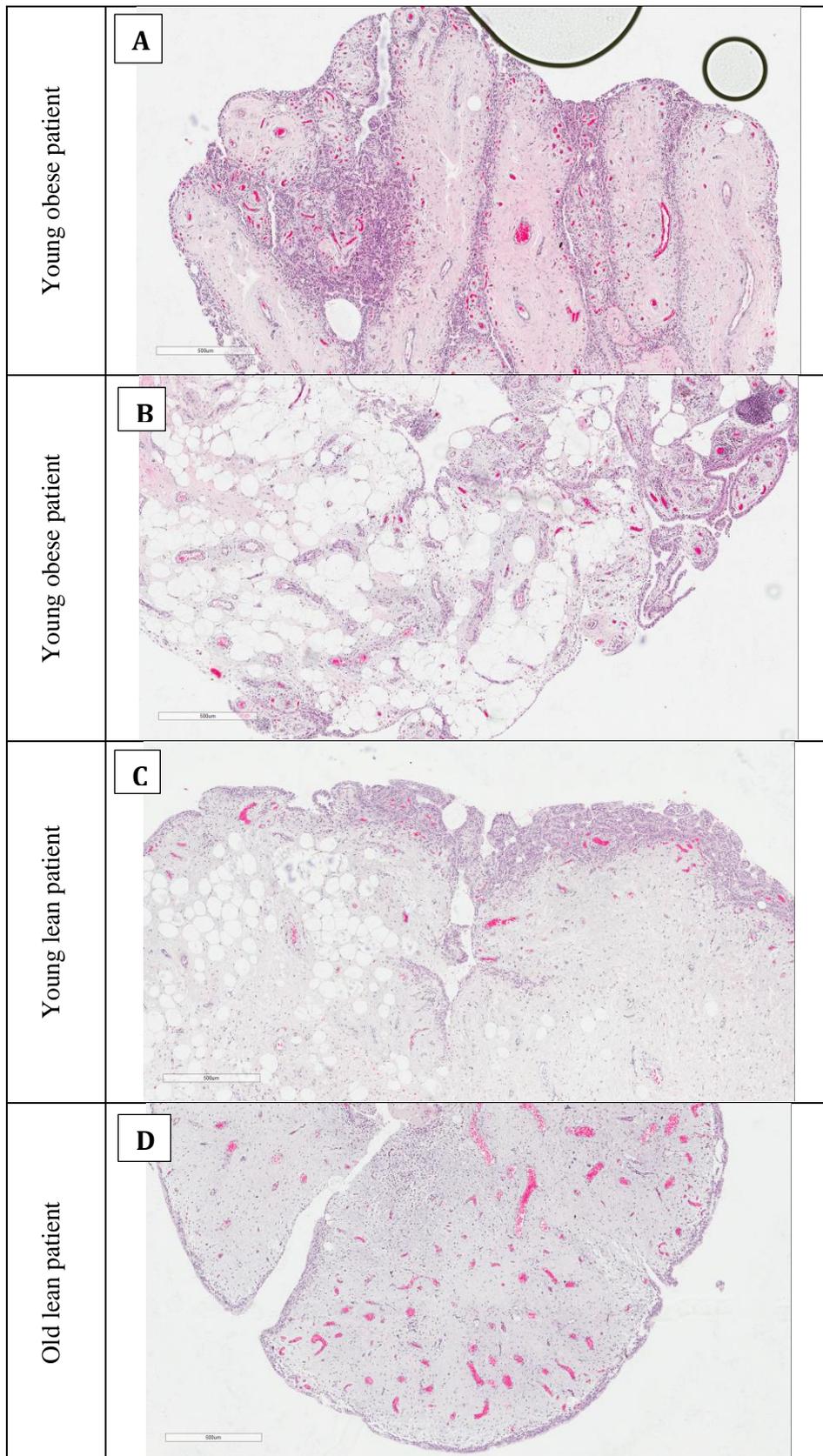
Figure 4. 3: young obese patient sample showing a florid synovitis with significant inflammatory cells infiltrations (A), another young obese patient showing a mix of fat and cellular components and also demonstrate synovitis (B), young lean patient showing evidence of synovitis(C), old lean patient with no evidence of synovitis(D).

4.3 Comparison of stain surface area

4.3.1 vWF

The DAB percentage was measured using ImageJ software as outlined in the methodology section (section 3.9.1.2). The vWF was measured in the synovial tissue and the fatty tissue for the whole slide section for each patient. The data were tested for normality and were found to be not normally distributed (Shapiro–Wilk test < 0.05). The Kruskall–Wallis test showed a significant difference across the groups in the synovial tissue. The significant values were readjusted using Dunn–Bonferroni *post hoc* analysis, which showed that the actual difference arises between group 3 and 1. On the other hand, the fatty tissue did not show statistical difference. Furthermore, the total (*i.e.* synovium and fat) percentage of vWF did not show any statistical difference. Although there was no statistical difference, there is a trend towards increased percentage in group 3 followed by group 2 and 1 (Table 4.3 and Figure 4.5). The same statistical procedures were applied to the other markers.

The measurement of vascularity using ImageJ software may not be very accurate because it relies on the percentage of the surface area of the vessels. The orientation of the vessels and the way they have been cut will affect the reading. This issue is demonstrated in the following IHC sections (Figure 4.4) of vWF of different groups. It is obvious from the slide sections that the number of actual vessels is greater in the obese patient's sample but because of the vessel orientation and shape the readings were significant for the old people. This was reflected in the significant difference in the synovial section in the old age group. This significant difference could be attributed to one outlier in group 3. To avoid this problem, the actual vessel counts in the hot spots were obtained.

The IHC samples of all the markers among the groups are shown in figure 4.33.
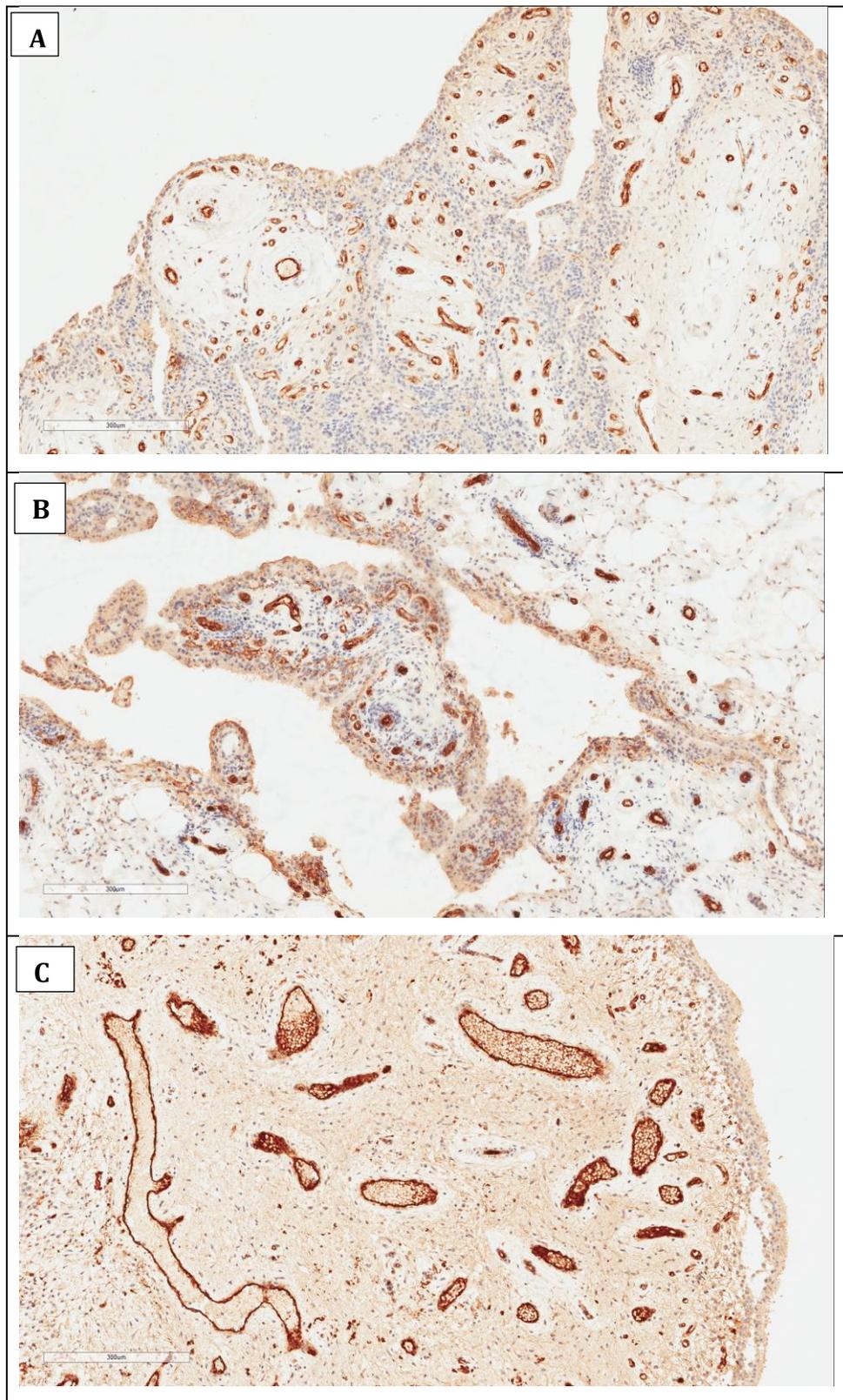
Figure 4. 4: *The vWF expression in different groups. Young obese patient sample shows multiple small microvessels (A), Young lean patient sample shows multiple small microvessels (B). Old lean patient sample shows vessels cut longitudinally representing a very big surface area and the actual count is less compared to other patients (C)*

Table 4.3: Percentage of vWF stain surface area

	Groups (N=17)			P value
	Young Obese (G1, n =6) (Age<60, BMI>30)	Young Lean (G2, n = 5) (Age<60, BMI<30)	Old Lean (G3, n = 6) (Age>70, BMI<30)	
	Median (SD)	Median (SD)	Median (SD)	
vWF Synovium	7.04(0.58)	7.12(1.06)	8.54(4.06)	**Across all groups : p= 0.029** [a] G1 = G2 : p =0.95 [b] **G1 < G3 : p = 0.024** [b] G2 < G3 : p = 0.40[b]
vWF Fat	7.01(0.87)	6.97(0.79)	7.71(3.60)	**Across all groups : p= 0.43**[a]
vWF Total	12.41(5.82)	14.04(1.85)	14.95(8.75)	**Across all groups : p= 0.293** [a]

Note. a = Kruskall–Wallis test.   b = Dunn–Bonferroni *post hoc* test.

It is important to note that group 3 had one case as an outlier, which could have made the difference significant.

1: Young Obese,   2:Young Lean,   3:Old Lean
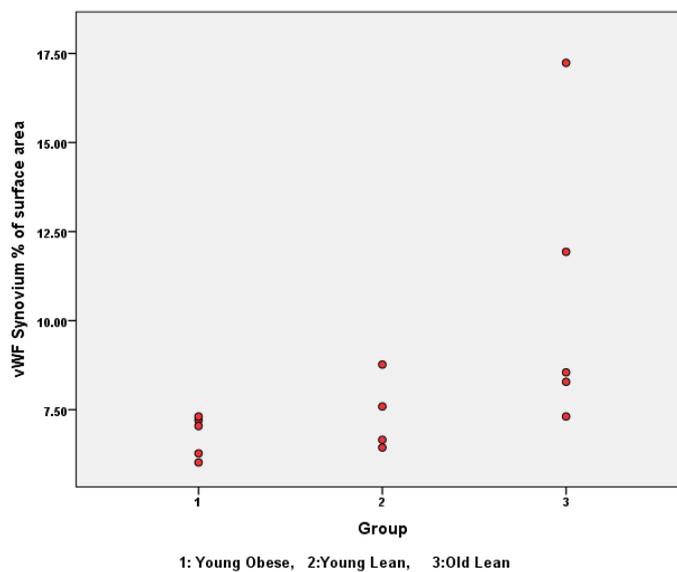
Figure 4.5: Percentage vWF synovial surface area.
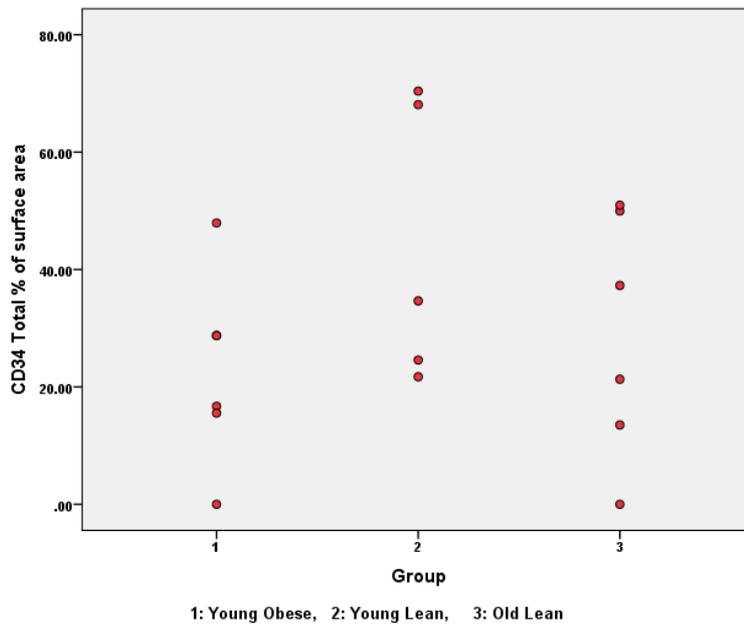
Figure 4.6: Percentage CD34 total surface area.

4.3.2 CD34

CD34 did not show any statistically significant difference in both synovial and fat sections across all the groups. The CD34 total also followed a similar trend when compared to vWF synovium, but not exactly the same (see Figures 4.5 and 4.6). Table 4.4 details the CD34 statistics. The following table (Table 4.4) shows examples the IHC staining of CD34.

Table 4. 4: Examples IHC staining of CD34

	Young Obese	Young Lean	Old Lean
CD34			

Table 4.5: Percentage of CD34 surface area

	Groups (N=17)			P value
	Young Obese (G1, n=6) (Age<60, BMI>30)	**Young Lean (G2, n=5) (Age<60, BMI<30)**	**Old Lean (G3, n = 6) (Age>70, BMI<30)**	
	Median (SD)	**Median (SD)**	**Median (SD)**	
CD34 Synovium	18.07 (6.22)	21.70(9.90)	24.47(9.82)	**Across all groups : p= 0.468[a]**
CD34 Fat	10.31(7.58)	12.95(14.20)	16.88(11.15)	**Across all groups : p= 0.692[a]**
CD34 Total	28.74(13.03)	34.65(23.64)	34.60(16.82)	**Across all groups : p= 0.445[a]**

Note. a = Kruskall–Wallis test.

4.3.3 CD68

CD68 did not show any statistical difference across groups. However, group 1 showed a greater increase in percentage when compared to the other groups with an increasing tendency going from group 3 towards group 1. In fact, in the fat section the difference is very big and very close to being significant (P = 0.059). There are outliers that could have made the difference not significant. The graphs in Figure 4.7 and 4.8 show this trend very clearly. The following table (table4.6) shows the IHC staining of CD68.

Table 4. 6: Examples of IHC staining of CD68

	Young Obese	Young Lean	Old Lean
CD68			
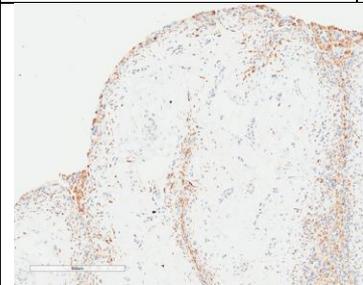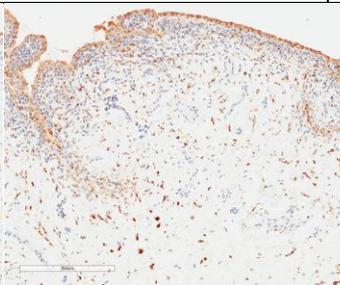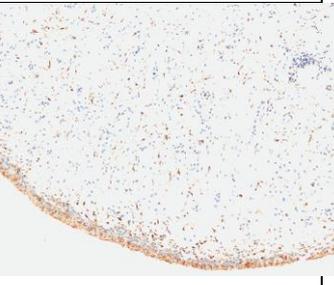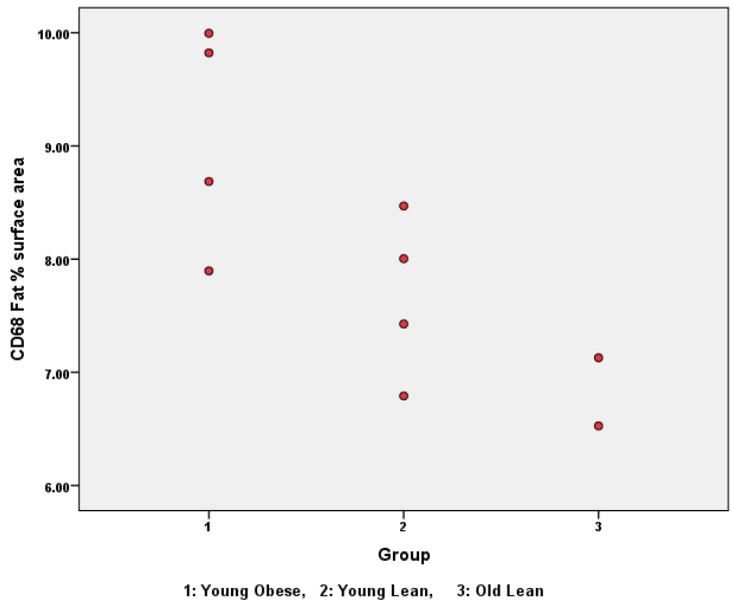
Figure 4.7: *Percentage of CD68 fat surface area.*
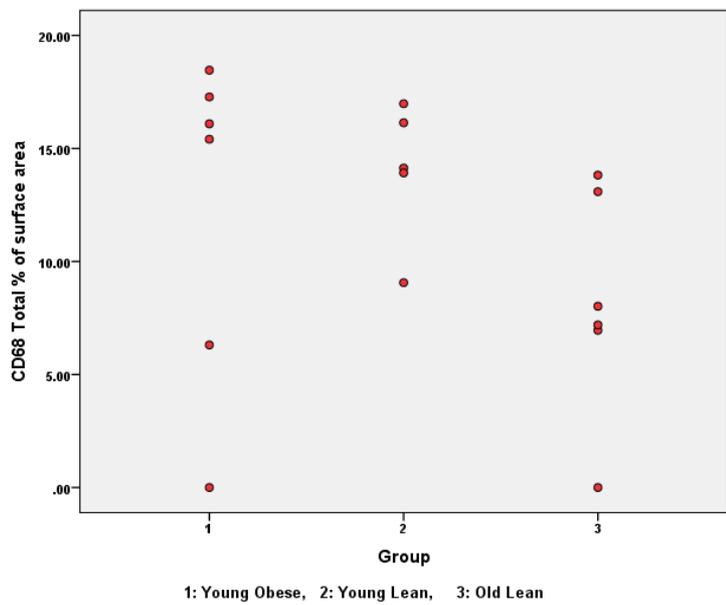
Figure 4.8: *Percentage of CD68 total surface area.*

Table 4.5 shows the statistical details for CD68. CD68's correlation with other markers, age and BMI will be analysed in the following sections.

Table 4.7: Percentage of CD68 stain surface area

	Groups (N=17)			P value
	Young Obese (G1, n =6) (Age<60, BMI>30)	**Young Lean (G2, n = 5) (Age<60, BMI<30)**	**Old Lean (G3, n = 6) (Age>70, BMI<30)**	
	Median (SD)	**Median (SD)**	**Median (SD)**	
CD68 Synovium	7.43(0.76)	7.90(0.97)	7.08(0.57)	**Across all groups : p= 0.264[a]**
CD68 Fat	9.10(0.99)	7.67(0.72)	6.82(0.42)	**Across all groups : p= 0.059[a]**
CD68 Total	14.71(4.83)	14.04(3.07)	9.81(3.35)	**Across all groups : p= 0.085[a]**

Note. a = Kruskall–Wallis test.

4.3.4 CD3

Although CD3 did not show any statistically significant differences across groups, it showed a trend of increased percentage in group 1 followed by group 2 and 3 in the synovial and total combined sections. The fat sections did not show any specific pattern. The boxplots in Figures 4.9 and 4.10 and Table 4.6 show the details of the results and how they are distributed. The following table (table 4.8) shows examples the IHC staining of CD3.

Table 4. 8: Examples of IHC staining of CD3

	Young Obese	Young Lean	Old Lean
CD3			
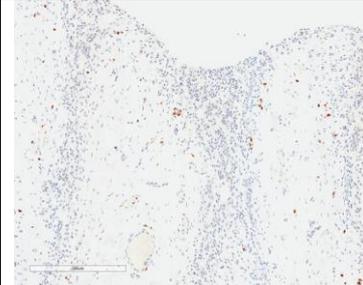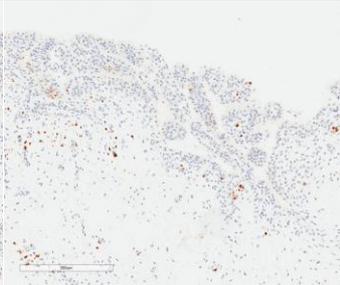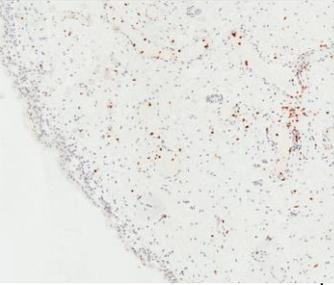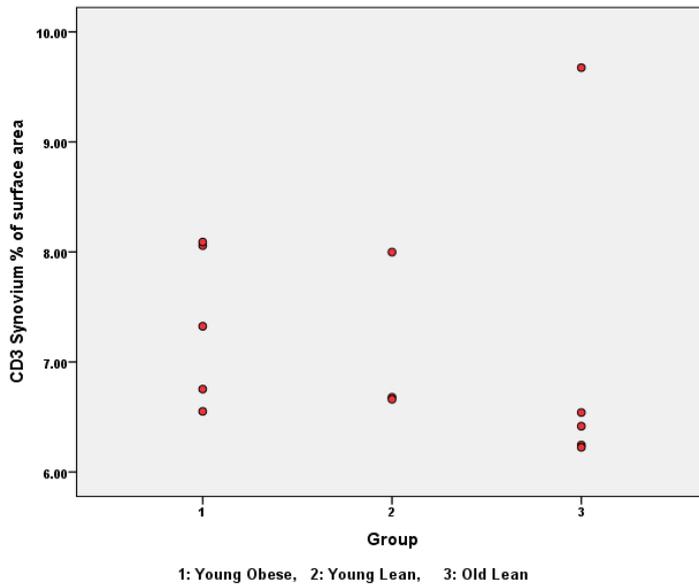
Figure 4.9: *Percentage of CD3 synovial surface area*
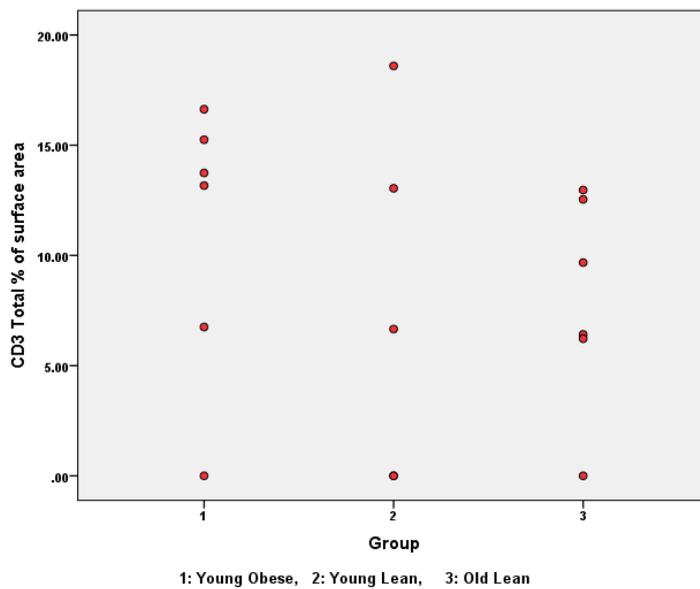
Figure 4.10: *Percentage of CD3 total surface area*

These graphs also shows that group 3 has got an outlier, which could affect the results. It shows the heterogeneity of the individuals even within the groups.

Table 4.9: Percentage of CD3 stain surface area

	Groups (N=17)			P value
	Young Obese (G1, n =6) (Age<60, BMI>30)	**Young Lean (G2, n = 5) (Age<60, BMI<30)**	**Old Lean (G3, n = 6) (Age>70, BMI<30)**	
	Mean (SD)	**Mean (SD)**	**Mean (SD)**	
CD3 Synovium	7.35(0.71)	7.11(0.76)	7.02((1.48)	**Across all groups : p= 0.192[a]**
CD3 Fat	7.19(0.95)	8.48(2.99)	6.36(0.08)	**Across all groups : p= 0.408[a]**
CD3 Total	13.10(3.80)	12.76(5.97)	9.56(3.22)	**Across all groups : p= 0.112[a]**

Note. a = Kruskall–Wallis test.

4.3.5 CD20

As for CD20, the across groups comparison did not show any statistically significant differences and there was no specific distribution pattern. However, there could be some hint of an increase in the synovial CD3 in the overall combined total sections. The details are shown in the graphs in Figures 4.11 and 4.12 and Table 4.7. The following table (table 4.10) shows examples the IHC staining of CD20.

Table 4. 10: Examples of IHC staining of CD20

	Young Obese	Young Lean	Old Lean
CD20			
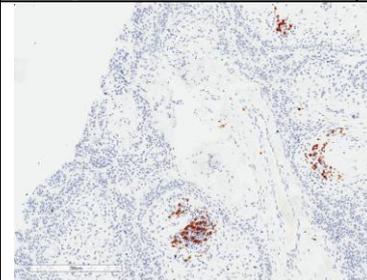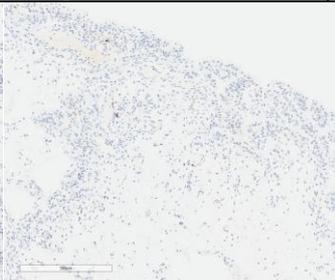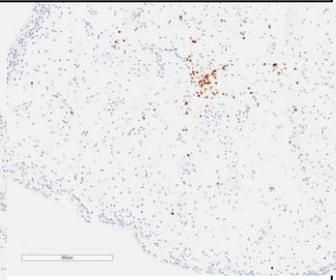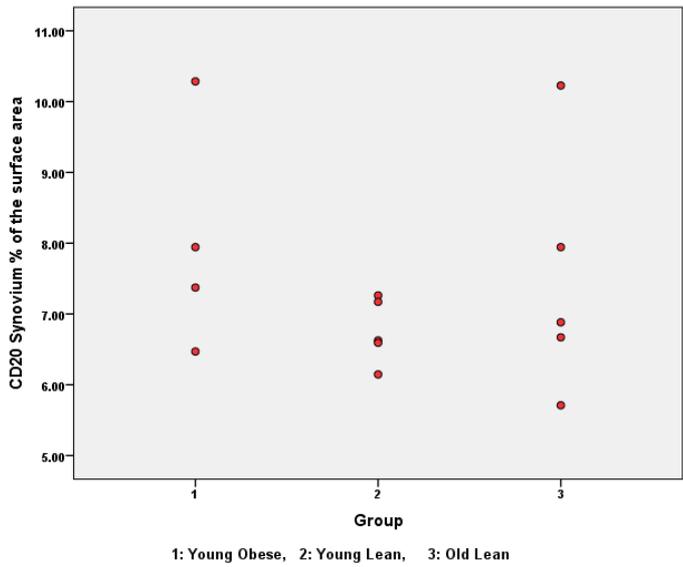
Figure 4.11: *Percentage of CD20 synovial surface area.*
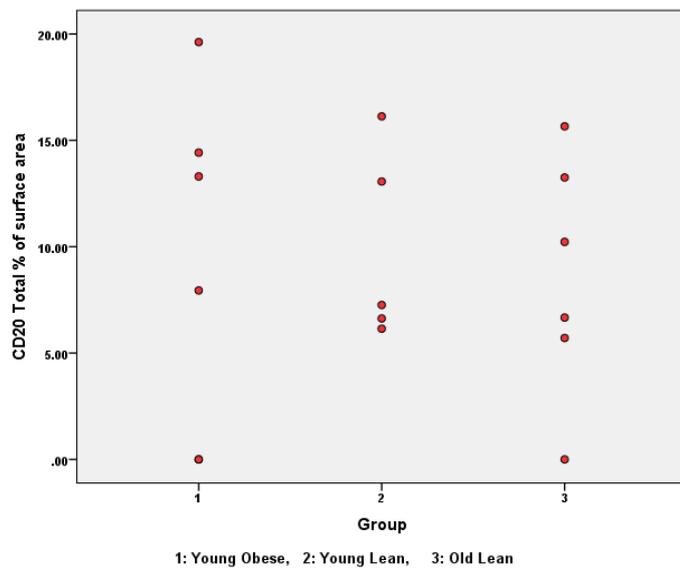
Figure 4.12: *Percentage of CD20 total surface area.*

The same outlier problem exists again for CD20. When looking into the data in detail, the outlier was not the same across the groups.

Table 4.11: Percentage of CD20 stain surface area

	Groups (N=17)			P value
	Young Obese (G1, n =6) (Age<60, BMI>30)	Young Lean (G2, n = 5) (Age<60, BMI<30)	Old Lean (G3, n = 6) (Age>70, BMI<30)	
	Mean (SD)	Mean (SD)	Mean (SD)	
CD20 Synovium	8.01(1.62)	6.75(0.45)	7.48(1.72)	Across all groups : p= 0.373[a]
CD20 Fat	7.73(1.38)	7.71(1.75)	7.04(0.94)	Across all groups : p= 0.700[a]
CD20 Total	13.82(4.78)	9.84(4.48)	10.30(4.23)	Across all groups : p= 0.295[a]

Note. a = Kruskall–Wallis test.

4.3.6 Vimentin

Vimentin showed statistical differences across the groups in the synovial, fat and total sections. Table 4.8 shows this in detail. Although there is significant difference across the groups, this difference changed following the Dunn–Bonferroni *post hoc* test readjustment. However, it is obvious that the vimentin marker is expressed more in the young patients (groups 1 and 2). The following table (table 4.12) shows examples of the IHC staining of Vimentin.

Table 4. 12: Examples of IHC staining of Vimentin

	Young Obese	Young Lean	Old Lean
Vim			
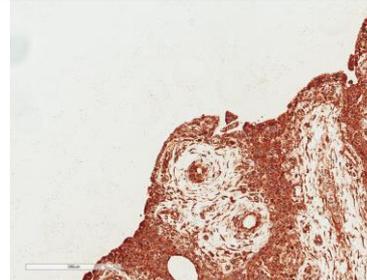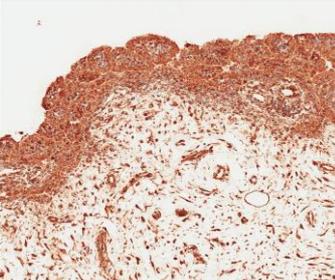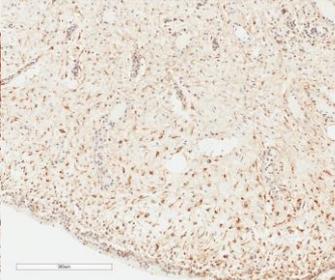
Table 4.13: Percentage of vimentin stain surface area

	Groups (N=17)			P value
	Young Obese (G1, n =6) (Age<60, BMI>30)	Young Lean (G2, n = 5) (Age<60, BMI<30)	Old Lean (G3, n = 6) (Age>70, BMI<30)	
	Mean (SD)	Mean (SD)	Mean (SD)	
Vim Synovium	15.40(6.21)	12.92(3.11)	6.37(0.31)	**Across all groups : p= 0.006 [a]** G1 = G2 : p =1.000 [b] **G1 > G3 : p = 0.013** [b] **G2 > G3 : p = 0.036[b]**
Vim Fat	11.08(6.47)	10.55(3.13)	6.31(0.22)	**Across all groups : p= 0.029[a]** G1 = G2 : p =1.000[b] G1 > G3 : p = 0.076 [b] G2 > G3 : p = 0.064[b]
Vim Total	24.27(13.21)	20.84(5.75)	10.58(3.46)	**Across all groups : p= 0.029[a]** G1 = G2 : p =1.000[b] **G1 > G3 : p = 0.044** [b] G2 > G3 : p = 0.058[b]

Note. a = Kruskall–Wallis test.   b = Dunn–Bonferroni *post hoc* test.

Figures 4.13 and 4.14 show the data distribution. They also highlight outliers; similarly to when looking at the previous results, it is not related to a particular group or patient.

1: Young Obese,   2: Young Lean,   3: Old Lean
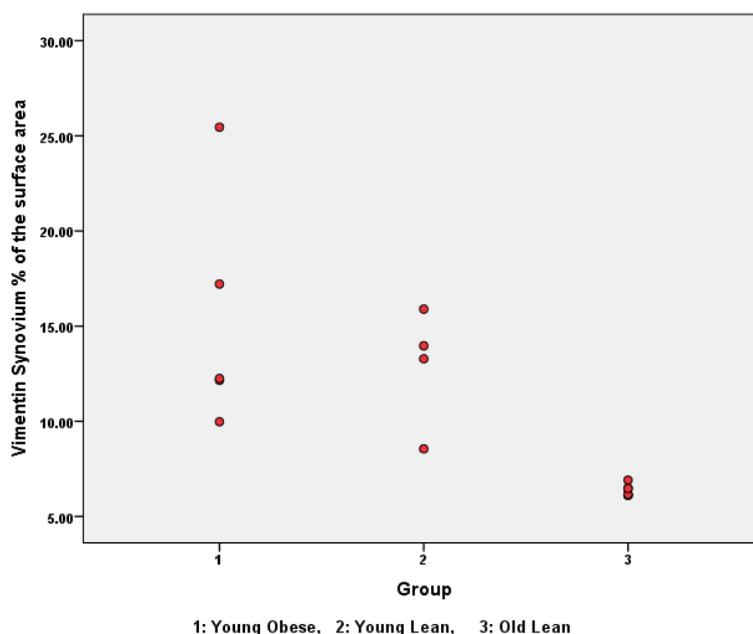
Figure 4.13*: Percentage of vimentin synovial surface area among groups.*
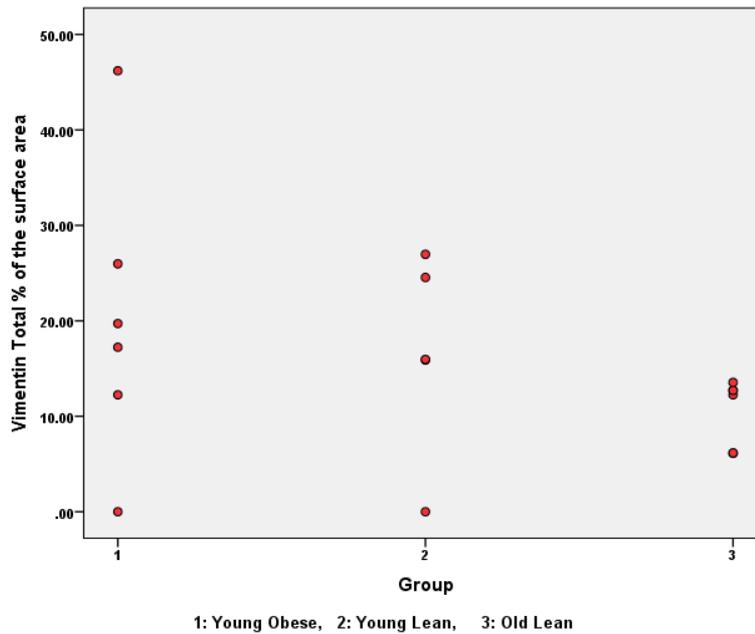
Figure 4.14: Percentage of vimentin total surface area among groups

4.3.7 Summary of ImageJ stain surface area measurements

The comparison of the markers among the groups showed significant differences for vWF synovium and vimentin only. ImageJ assessment of vascularity is not accurate, as explained in Section 4.3.1. Vimentin showed a significant difference between groups, being highest in group 1 and 2. Although not significant, the difference of the other markers showed a trend among the groups. They were higher in the obese young patients followed by young lean and old lean patients. These findings suggest an increased inflammatory response in the young obese patients. The total vascular markers (*i.e.* vWF and CD34) did not show any statistically significant difference.

4.4 Comparison of other microvascular measurements

Other important measurements were obtained and are listed in Table 4.9.

4.4.1 Vascular density in the synovial intima

The data showed no significant difference across groups for vascular density in the synovial intima (p = 0.915). The boxplot chart is shown in Figure 4.15.

Table 4.14: Comparison of vascular measurements (VDS= vascular density in synovium, SMC= smooth muscle cell)

	Groups (N=17)			P value
	Young Obese (G1, n =6) (Age<60, BMI>30)	**Young Lean (G2, n = 5) (Age<60, BMI<30)**	**Old Lean (G3, n = 6) (Age>70, BMI<30)**	
	Median (SD)	**Median (SD)**	**Median (SD)**	
VDS	52.00(26.58)	45.00(4.42)	41.00(9.52)	**Across all groups : p= 0.915[a]**
Vessels with SMC	10.00(2.70)	7.00(2.06)	13.00(4.18)	**Across all groups : p= 0.013[a]** G1 > G2 : p =0.537[b] **G1 < G3 : p = 0.283** [b] G2 < G3 : p = 0.010[b]
Vessels with no SMC	42.00(24.14)	36.50(4.79)	30.00(8.55)	**Across all groups : p= 0.305[a]**
VD Fat	28.00(19.52)	29.00(3.46)	23.00(10.45)	**Across all groups : p= 0.127[a]**
Vessel distance	49.78(14.54)	50.17(4.54)	36.66(4.16)	**Across all groups : p= 0.023[a]** G1 = G2 : p =1.000[b] **G1 > G3 : p = 0.038** [b] G2 > G3 : p = 0.089[b]
Vessel wall thickness	16.05(5.86)	15.88(4.20)	11.85(3.47)	**Across all groups : p= 0.195[a]**
Mean fat cell diameter	84.25(11.19)	64.41(6.74)	50.46(8.09)	**Across all groups : p= 0.002[a]** G1 > G2 : p =0.221[b] **G1 > G3 : p = 0.001** [b] G2 > G3 : p = 0.525[b]

Note. a = Kruskall–Wallis test.   b = Dunn–Bonferroni *post hoc* test.

Although the median suggests a decreasing trend from group 1 to group 3, it is difficult to draw a meaningful conclusion because of the outliers in groups 1 and 3.
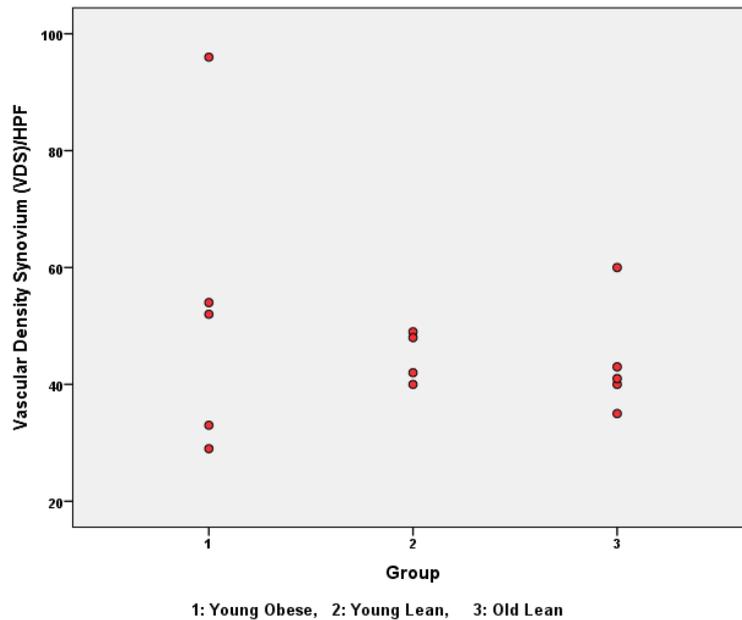
Figure 4.15: *Vascular density in the synovial intimal layer per group*

4.4.1.1 Intima vessels with smooth muscle cells layer

The intimal vascular density was further evaluated for the presence of smooth muscle cells in the vascular wall. The data is shown in Table 4.9. It is interesting to see that the presence of SMC is significantly different across the groups (p = 0.013). This difference was noted between groups 1 and 3 according to the Dunn–Bonferroni *post hoc* test readjustments. It showed that old lean people have increased numbers of vessels with smooth muscle cells, as shown in Figure 4.16.

4.4.1.2 Intima vessels with no smooth muscle cells layer

The number of intimal vessels that lack the SMC layer were also counted to relate it to the overall density. There was no significant difference across groups. However, this is an important finding. The boxplot in Figure 4.17 shows a trend of increased number of vessels lacking an SMC layer in the obese group, followed by the lean young and then the lean old group.
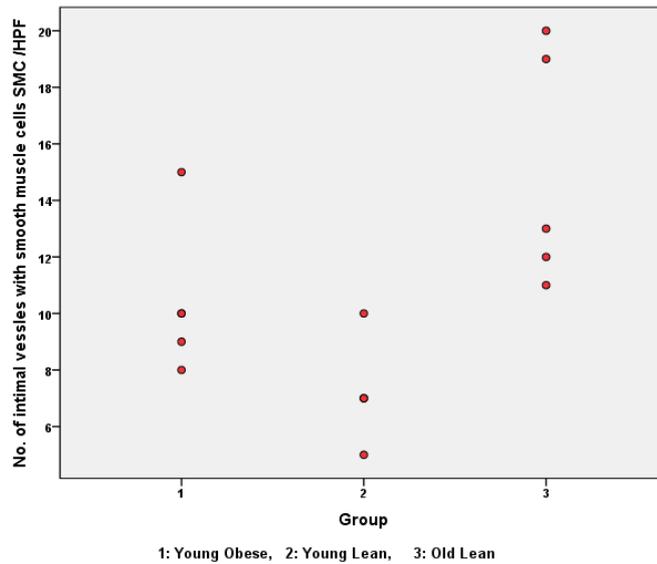
Figure 4.16: Intimal vessels with smooth muscle cells.
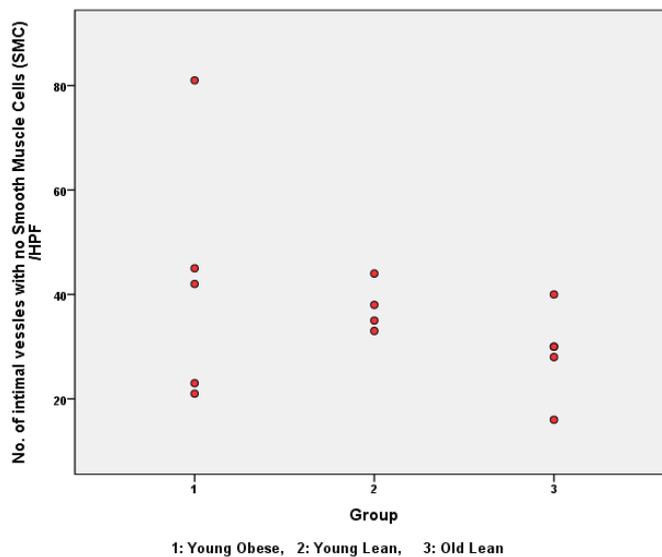
Figure 4.17: Intimal vessels lacking smooth muscle cells.

4.4.2 Vascular density in the fat section

There was no significant difference across groups for the vascular density in the fat sections **(p = 0.127)**. The statistical details are shown in Table 4.9. Figure 4.18 gives a visual representation of the distribution. There was a trend of increased vascular density in the fatty sections of the obese people compared to in the other groups.
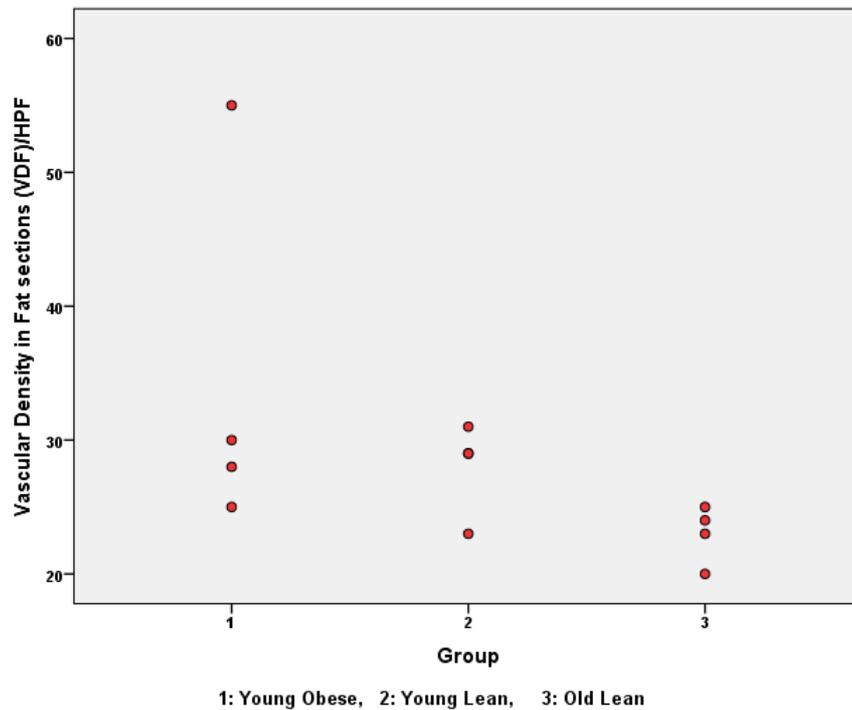
Figure 4.18: *Vascular density in the fat area.*

4.4.3 Mean vessel distance from the surface of intimal synovium

The mean distance of the vessels from the synovial surface is outlined in Table 4.9. A significant difference across groups is demonstrated (**p = 0.023**). This significance is between groups 1 and 3, where group 1 shows an increased distance. Group 2 also has showed an increased distance when compared to group 3 but the Dunn–Bonferroni *post hoc* test readjustments gave a p value of (**p = 0.089**). The boxplot is shown in Figure 4.19.
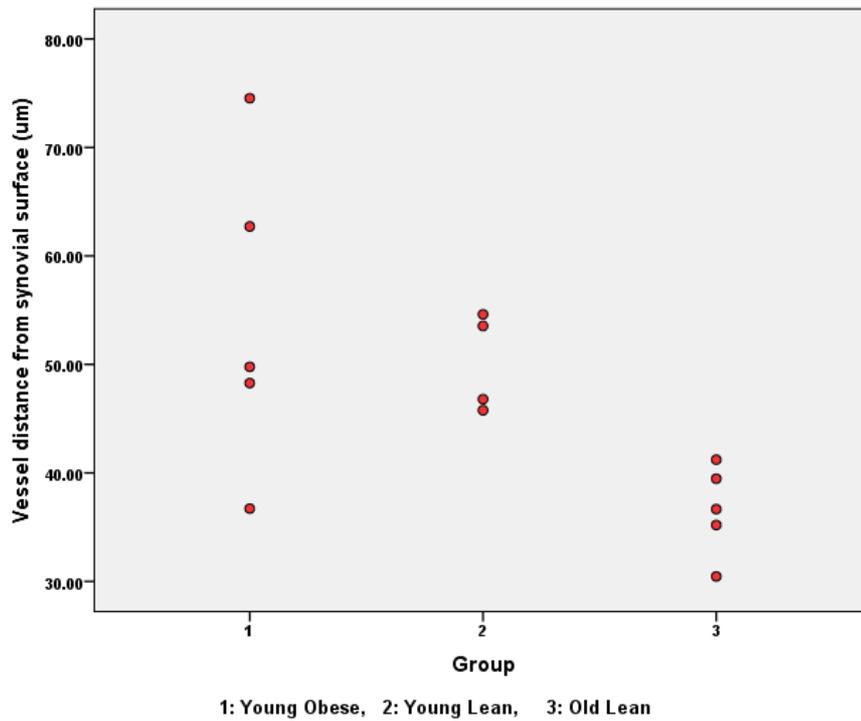
Figure 4.19: *Mean distance of the intimal synovial vessels from the synovial surface.*

4.4.4 Vessel wall thickness

The largest vessels in each section were measured and the mean calculated to see if the wall thickness differed among the groups. Across group comparison did not show any significant difference. However, there is a trend of increasing vessel wall thickness in the obese young patients followed by the lean young and lean old people, as shown in Figure 4.20.
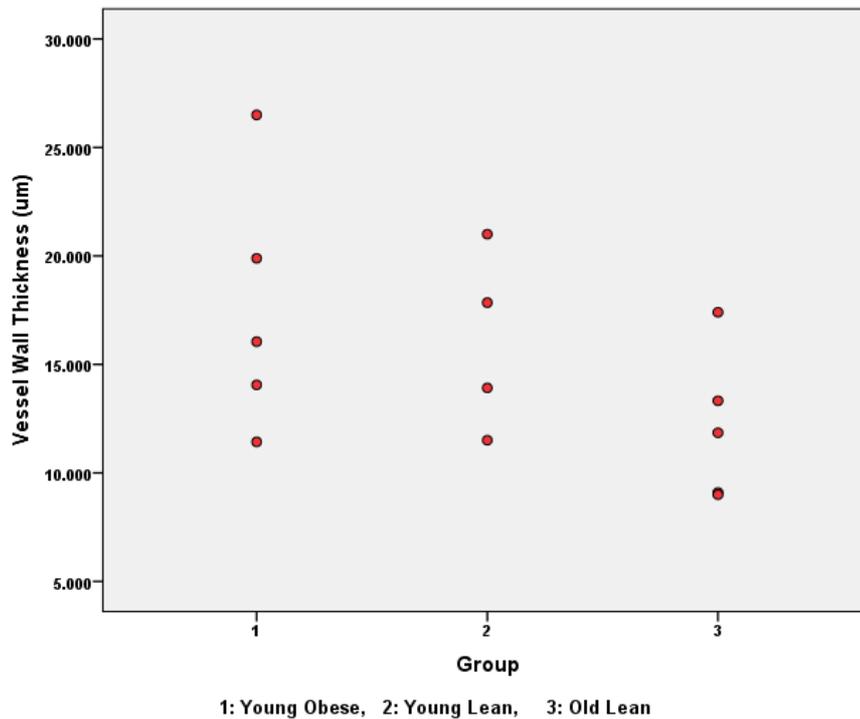
Figure 4.20: Vessel wall thickness.

4.4.5 Summary of microvascular measurements

The vascular measurements did not show any significant difference among the groups, except for the vessels with a smooth muscle layer in the synovial intima. Vessels in the synovial intima with a smooth muscle layer are significantly more abundant in the old age group (group 3), whereas the number of vessels that lack a smooth muscle layer were higher in the young obese group (group 1). Another trend of increased vascular density was observed in the fat sections. The young groups (groups 1 and 2) showed an increase in the distance of the nearest vessels to the synovial surface as well as the wall thickness. These results show that there are changes in the microcirculation in the synovium of these different groups. The increased vessel wall thickness (hyperplasia) is an important difference along with the vascular distance from the synovial surface.

4.5 Comparison of fat cell diameter in relation to other markers

4.5.1 Mean fat cell diameter

The data on the fat dimensions revealed a significant difference across groups (p = **0.002, Table 4.9)**. This difference is observed between groups 1 and 3 (**G1 > G3: p = 0.001**). The box plot (Figure 4.21) shows some obvious differences between the groups, but this was not detected statistically. To assess how fat affects other markers, the results were divided into groups based on fat cell diameter (smaller and larger) and the Mann–Whitney U test applied across all the markers, irrelevant of groups. This comparison is shown in the following sections.
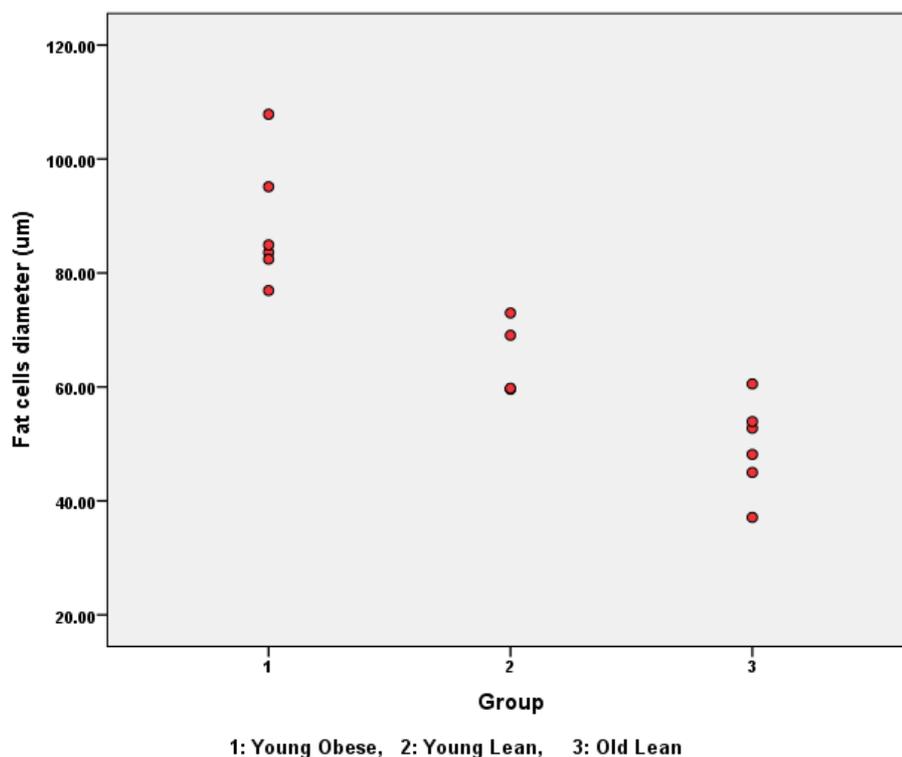
Figure 4.21: Mean fat cell diameter.
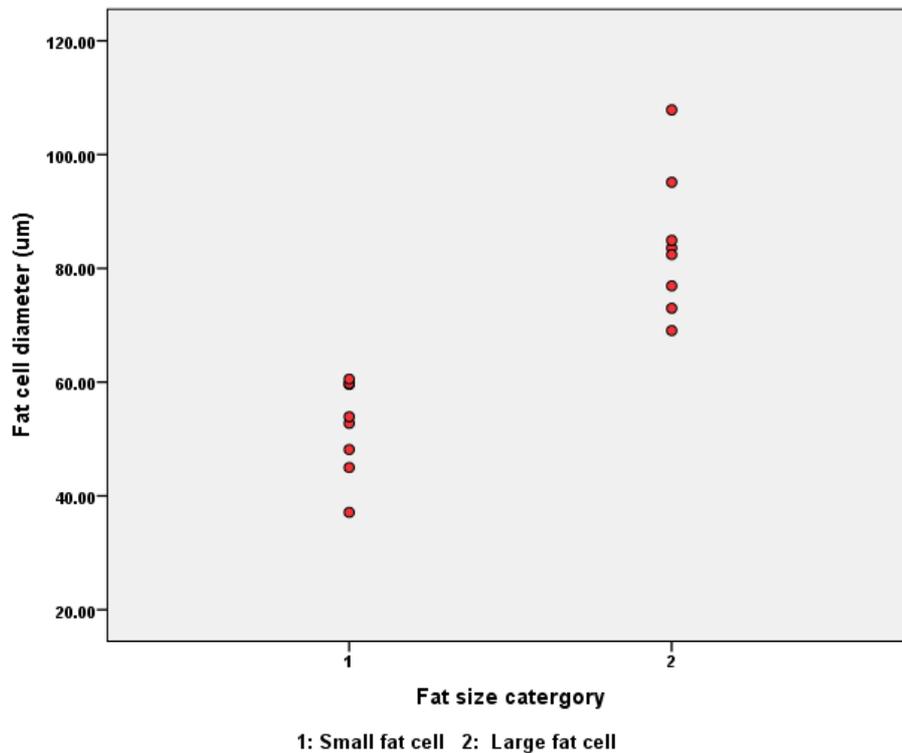
Figure 4.22: The two fat categories (1= small, 2= large).

4.5.2 Mean fat cell diameter comparison with other markers

The patients were divided into small and large diameter fat cell groups after taking the average size of all the measurements and separating the groups into small size for those below the average and big size for those above the average. There was an equal distribution with eight patients in each group (one sample was not included as it did not contain fatty tissue; the results are shown in a box plot in Figure 4.22. There was only one patient who lied very close to the separation line. This assessment was done to see if the actual local fat burden as indicated by the fat cell diameter will affect the markers distribution regardless of the patient's age and BMI.

Table 4.10 shows the variables that exhibited a significant difference between the small and big fat cell diameter group.

Table 4.15: The variables with significant difference between the small and large fat cell groups (S= synovium, VDF= vascular density in fat, SMC= smooth muscle cell)

	vWF S	CD68 Total	CD3 Total	CD20 Total	Vimentin Total	Vessel distance	VDF	VD with SMC
P value	0.004	0.035	0.016	0.046	0.014	0.046	0.028	0.021

The difference with the synovial vWF was significant. The vWF was higher in the small size fat cells. The reason for this difference was explained in Section 4.3.1. A similar difference was observed with the vessels with a smooth muscle layer where an increase in size of the fat cells correlated with a decrease in the number of vessels with a smooth muscle layer. The boxplots in Figures 4.23 and 4.24 show this comparison. On the other hand, CD68 total (macrophage marker), CD3 total (T-cell marker), CD20 total (B-cell marker), vimentin synovium (cytoskeleton protein), vimentin fat, vimentin total, intimal vessel distance from the synovial surface and vascular density in the fat section showed an increase with increased size of the fat cells.
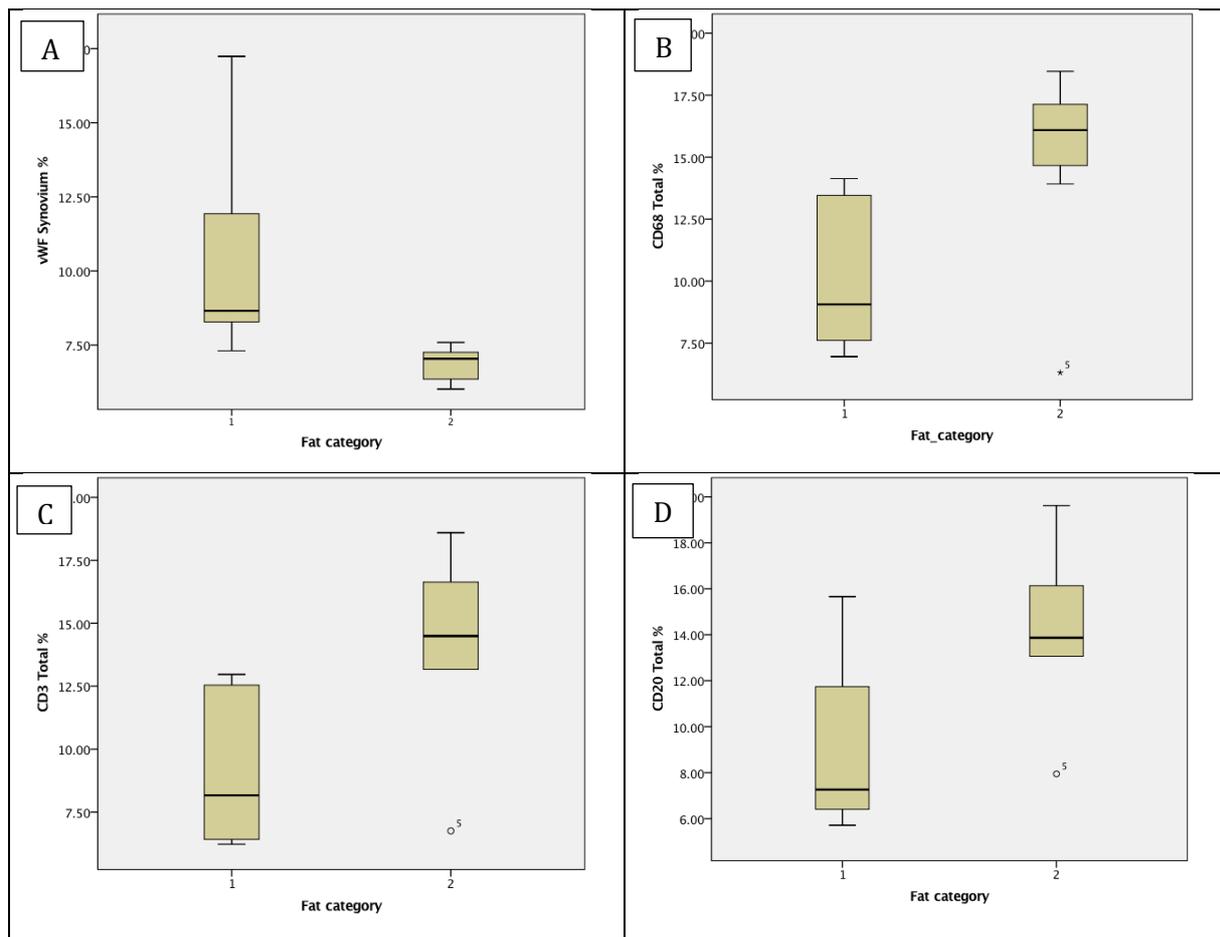
Figure 4.23: *The boxplots shows comparison of fat cell size (1= small fat cell group, 2= large fat cell group) with synovial vWF (A), CD68 total (B), CD3 total (C) and CD20 total (D).*
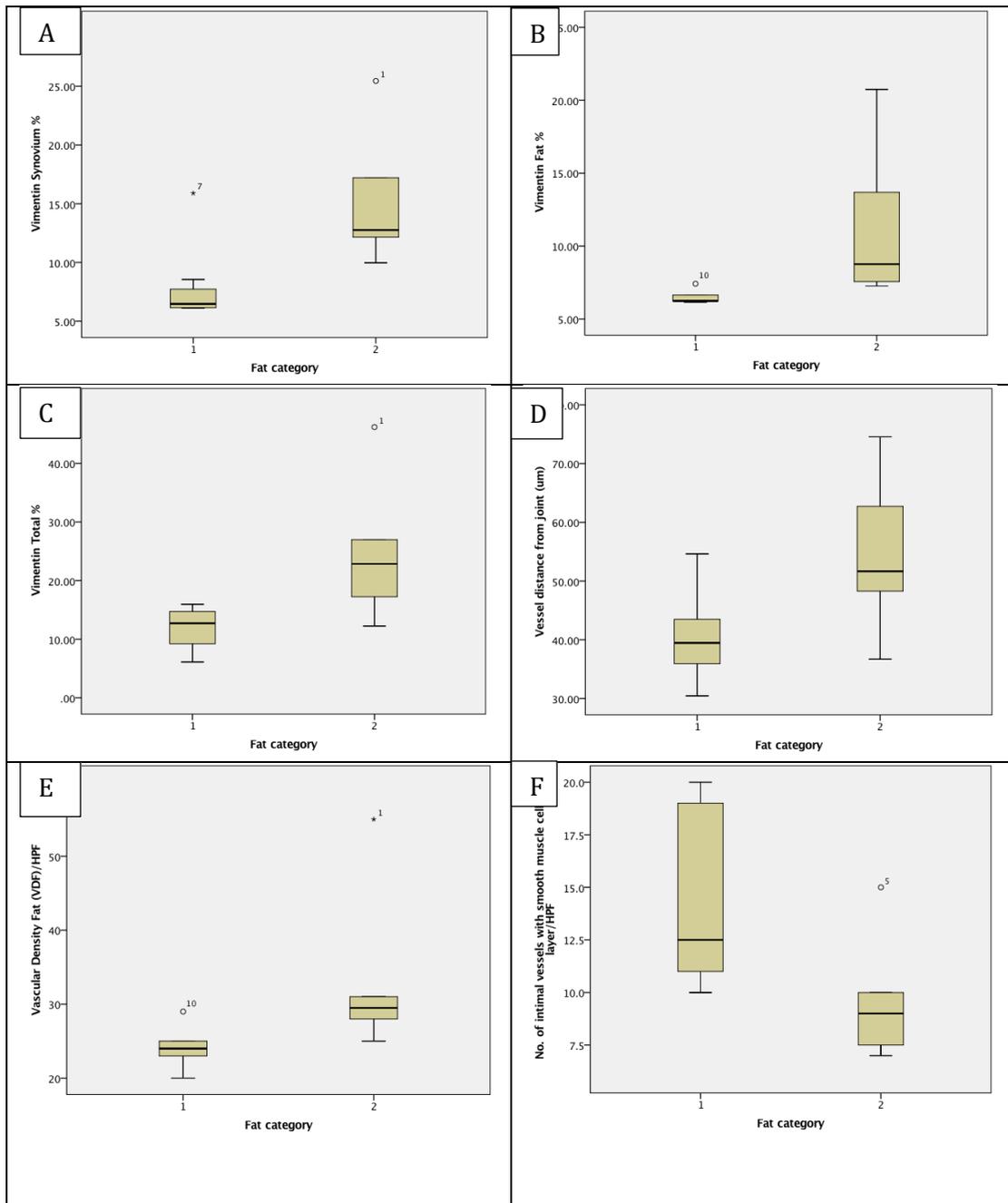
Figure 4.24: *The boxplots shows comparison of fat cell size (1= small fat cell group, 2= large fat cell group) with vimentin synovium (A), vimentin fat (B), vimentin total (C), intimal vessel distance from synovial surface (D), vascular density in fat sections (E), number of vessels with smooth muscle layer (F).*

4.5.3 Summary of fat cell comparison

Fat cell size affected several markers as outlined above. The increase in fat cell size correlated with an increase in the inflammatory markers of macrophages, T cells, B cells and fibroblasts. Moreover, the number of vessels in fatty sections increased with increased fat cell size. These results suggest that an increase in the fat cell size has a major role in the inflammatory response.

4.6 Correlations

Tables 4.11, 4.12, 4.13, 4.14 and 4.15 depict the data and details of correlations. All the markers have been correlated with each other and with other parameters. The correlations are summarised as follows:

4.6.1 Age and inflammatory markers in the synovial sections

Age had a very significant negative correlation with BMI, CD3 and vimentin in the synovium sections. The relationship between age and BMI has been explained earlier and it is important to see how the synovium differs among the groups with different BMIs. In the synovial sections, CD3 and vimentin showed a significant negative correlation with age. Although not significant, there was a negative correlation with other markers, including CD68 and CD20. These findings suggest that the inflammatory response is lower in the synovium of the old age group. For details of the results, refer to Table 4.11.

Table 4.16: Table of correlation of Age and BMI with all the markers measured in the synovial sections.

		Age	BMI	vWF S	CD34 S	CD68 S	CD3 S	CD20 S	Vim S
Age	Correlation Coefficient	1.000	-0.754**	0.564*	0.275	-0.338	-0.646*	-0.209	-0.792**
	P value	.	0.000**	0.036*	0.322	0.219	0.017*	0.473	0.000
BMI	Correlation Coefficient	-0.754**	1.000	-0.459	-0.154	-0.154	0.484	0.389	0.654**
	P value	0.000**	.	0.098	0.585	0.585	0.094	0.169	0.008
vWF S	Correlation Coefficient	0.564*	-0.459	1.000	0.411	-0.481	-0.324	-0.343	-0.720**
	P value	0.036*	0.098	.	0.144	0.081	0.280	0.276	0.006**
CD34 S	Correlation Coefficient	0.275	-0.154	0.411	1.000	-0.479	-0.170	-0.220	-0.226
	P value	0.322	0.585	0.144	.	0.071	0.578	0.471	0.436
CD68 S	Correlation Coefficient	-0.338	-0.154	-0.481	-0.479	1.000	0.577*	0.121	0.640*
	P value	0.219	0.585	0.081	0.071	.	0.039*	0.694	0.014*
CD3 S	Correlation Coefficient	-0.646*	0.484	-0.324	-0.170	0.577*	1.000	0.627*	0.615*
	P value	0.017*	0.094	0.280	0.578	0.039*	.	0.039*	0.025*
CD20 S	Correlation Coefficient	-0.209	0.389	-0.343	-0.220	0.121	0.627*	1.000	0.238
	P value	0.473	0.169	0.276	0.471	0.694	0.039*	.	0.457
Vim S	Correlation Coefficient	-0.792**	0.654**	-0.720**	-0.226	0.640*	0.615*	0.238	1.000
	P value	0.000**	0.008**	0.006**	0.436	0.014*	0.025*	0.457	.

*Significant correlation at 0.05 ** Significant correlation at 0.01

4.6.2 Age and inflammatory markers in the total sections

The inflammatory markers (*i.e.* CD3, CD20, CD68 and vimentin) combined in the synovium and the fat sections showed similar correlations when compared to the synovial sections. This kind of correlation suggests that the older patients are not showing a significant inflammatory response despite having the same grade of OA as compared to the young group of patients, as shown in Table 4.14.

4.6.3 Age and vascular measurements

A significant positive correlation of age was observed with the synovial vWF marker. There was also a positive correlation between age and CD34 but not significant. Although age had a positive correlation with vWF and CD34, it was correlated negatively with the other vascular measurements. Vascular density in the synovium (VDS) and fat (VDF) sections and the vascular density of the vessels that lack a smooth muscle layer (vessels with no SMC) correlated negatively with age. In addition, a negative correlation was observed with the vascular wall thickness, vessel distance from synovial surface and fat cell diameter. On the other hand, there was a positive correlation between age and the vessels that had a smooth muscle layer. The accuracy of the vWF and CD34 results obtained by ImageJ has been explained in Section 4.3.1 and might explain why there was discrepancy between the vWF and CD34 measured by the ImageJ software and the other vascular measurements. Table 4.12 shows the statistical correlations and data in detail.

Table 4.17: Table of correlation of age, BMI and all the markers in the synovial sections with all the vascular measurements

		MVDS	MVDF	MVD (SMC)	MVD (No SMC)	Wall thickness	Vessel distance	Fat cell diameter
Age	Correlation Coefficient	-0.042	-0.479	0.417	-0.279	-0.437	-0.644*	**-0.738****
	P value	0.886	0.083	0.138	0.334	0.119	0.013*	**0.001****
BMI	Correlation Coefficient	0.048	0.252	-0.150	0.183	0.319	0.407	**0.724****
	P value	0.869	0.385	0.608	0.532	0.267	0.149	**0.002****
vWF S	Correlation Coefficient	-0.196	-0.397	**0.537***	-0.381	-0.253	-0.456	**-0.742****
	P value	0.502	0.159	**0.048***	0.179	0.405	0.117	**0.004****
CD34 S	Correlation Coefficient	0.172	0.252	0.175	0.084	0.156	-0.095	-0.073
	P value	0.557	0.385	0.551	0.776	0.594	0.47	0.805
CD68 S	Correlation Coefficient	-0.172	0.325	**-0.754****	0.051	0.415	0.262	0.068
	P value	0.557	0.258	**0.002****	0.864	0.140	0.336	0.817
CD3 S	Correlation Coefficient	0.170	0.326	-0.464	0.380	**0.676***	**0.687****	0.462
	P value	0.578	0.277	0.110	0.201	**0.011***	**0.010****	0.131
CD20 S	Correlation Coefficient	0.343	-0.011	0.224	0.301	0.448	0.168	0.154
	P value	0.275	0.974	0.484	0.341	0.145	0.602	0.616
Vim S	Correlation Coefficient	0.253	0.497	**-0.724****	0.514	0.508	**0.666****	**0.727****
	P value	0.405	0.084	**0.005****	0.072	0.064	**0.009****	**0.003****

*Significant correlation at 0.05 ** Significant correlation at 0.01

4.6.4 BMI and inflammatory markers in the synovial sections

The relationship between BMI and the inflammatory markers was the opposite of the age relationship. In the synovial sections, CD3, CD20 and vimentin were positively correlated with increased BMI. Vimentin had a very significant correlation. Interestingly, CD68 was correlated negatively with the BMI. However, when looking

at the total CD68, the relationship followed the other markers. Refer to Table 4.11 for further details.

4.6.5 BMI and inflammatory markers in the total sections

The inflammatory markers in the total sections showed similar correlations when compared to the synovial sections. This is an indication that increasing weight is correlated with increasing inflammatory response. Details are shown in Table 4.14.

Table 4.18: Table of correlation of the vascular measurements with each other

		MVDS	MVDF	MVD (SMC)	MVD (No SMC)	Wall thickness	Vessel distance	Fat cell diameter
MVDS	Correlation Coefficient	1.000	0.014	0.200	**0.925****	0.110	0.385	0.228
	P value		0.961	0.493	**0.000****	0.721	0.194	0.453
MVDF	Correlation Coefficient	0.014	1.000	-0.529	0.158	**0.776****	0.395	0.502
	P value	0.961		0.052	0.590	**0.002****	0.182	0.080
MVD (SMC)	Correlation Coefficient	0.200	-0.529	1.000	-0.146	-0.453	-0.464	-0.373
	P value	0.493	0.052		0.618	0.120	0.110	0.209
MVD (No SMC)	Correlation Coefficient	0.925	0.158	-0.146	1.000	0.217	**0.597***	0.465
	P value	0.000	0.590	0.618		0.476	**0.031***	0.109
Wall thickness	Correlation Coefficient	0.110	**0.776****	-0.453	0.217	1.000	**0.547***	0.489
	P value	0.721	**0.002****	0.120	0.476		**0.043***	0.09
Vessel distance	Correlation Coefficient	0.385	0.395	-0.464	**0.597***	**0.547***	1.000	**0.648***
	P value	0.194	0.182	0.110	**0.031***	**0.043***		**0.017***
Fat cell diameter	Correlation Coefficient	0.228	0.502	-0.373	0.465	0.489	**0.648***	1.000
	P value	0.453	0.080	0.209	0.109	0.09	**0.017***	

*Significant correlation at 0.05 ** Significant correlation at 0.01

4.6.6 Other correlations

4.6.6.1 CD68 vs. age

Although there was a negative correlation between CD68 and age, this correlation was weak with a correlation coefficient of -0.314 (p = 0.254). See Tables 4.14 and 4.15 for details.

4.6.6.2 CD68 vs. BMI

BMI showed a positive correlation with CD68. This correlation was weak. However, it showed a significant positive correlation with fat cell diameter. See Tables 4.14 and 4.15 for details.

4.6.6.3 CD68 vs. vascular measurements

There was a very strong significant correlation between CD68 and the vascular density in the fat sections. There was also a weak positive correlation observed between MVDS and CD68. Vascular wall thickness and fat cell diameter were correlated significantly. This relationship is expected because, as the vascularity increases, the number of inflammatory cells like macrophages increase. On assessment of the MVD with SMC, it was found to have a significant negative correlation. So, macrophages increase in number with increased vascularity. See Tables 4.14 and 4.15 for details.

4.6.6.4 Vimentin vs. age

Vimentin exhibited a similar pattern of correlation to CD68. However, the strength of the correlation was different. There was a very strong significant negative correlation between vimentin and age. See Tables 4.14 and 4.15 for details.

4.6.6.5 Vimentin vs. BMI

Although not significant, there was a positive correlation between vimentin and BMI. The fat cell diameter showed a significant positive correlation with vimentin. See Tables 4.14 and 4.15 for details.

4.6.6.6 Vimentin vs. vascular measurements

The vascular measurements showed a positive correlation with vimentin, except for MVD with SMC, which showed the complete opposite. These findings are in agreement with the CD68 findings in relation to the vascular parameters. See Tables 4.14 and 4.15 for details.

Table 4.19: Correlation of age and BMI with all the markers in the whole tissue sections

		Age	BMI	vWF Total	CD34 Total	CD68 Total	CD3 Total	CD20 Total	Vim Total
Age	Correlation Coefficient	1.000	-0.754**	0.071	0.066	-0.314	-0.554*	-0.202	-0.588*
	P value		0.000**	0.810	0.814	0.254	0.049*	0.448	0.021*
BMI	Correlation Coefficient	-0.754**	1.000	-0.015	-0.029	0.232	0.357	0.284	0.461
	P value	0.000**		0.958	0.919	0.405	0.231	0.326	0.084
vWF Total	Correlation Coefficient	0.071	-0.015	1.000	0.908**	0.204	0.104	0.042	0.242
	P value	0.810	0.958		0.000**	0.483	0.734	0.897	0.426
CD34 Total	Correlation Coefficient	0.066	-0.029	0.908**	1.000	0.296	0.071	0.154	0.257
	P value	0.814	0.919	0.000**		0.283	0.817	0.616	0.375
CD68 Total	Correlation Coefficient	-0.314	0.232	0.204	0.296	1.000	0.857**	0.495	0.912**
	P value	0.254	0.405	0.483	0.283		0.000**	0.086	0.000**
CD3 Total	Correlation Coefficient	-0.554*	0.357	0.104	0.071	0.857**	1.000	0.818**	0.835**
	P value	0.049*	0.231	0.734	0.817	0.000**		0.002**	0.000**
CD20 Total	Correlation Coefficient	-0.202	0.284	0.042	0.154	0.495	0.818**	1.000	0.427
	P value	0.448	0.326	0.897	0.616	0.086	0.002**		0.167
Vim Total	Correlation Coefficient	-0.588*	0.461	0.242	0.257	0.912**	0.835**	0.427	1.000
	P value	0.021*	0.084	0.426	0.375	0.000**	0.000**	0.167	.

*Significant correlation at 0.05 ** Significant correlation at 0.01

4.6.6.7 CD3 and CD20 vs. age

CD3 showed a significant negative correlation with age. On the other hand, CD20 showed a weak negative correlation with age. See Tables 4.14 and 4.15 or details.

4.6.6.8 CD3 and CD20 vs. BMI

In contrast to age, they showed a positive correlation with BMI. However, it was not a significant correlation. See Tables 4.14 and 4.15 for details.

4.6.6.9 CD3 and CD20 vs. vascular measurements

Both markers exhibited a similar type of relationship with vascular measurements as that for CD68 and vimentin, except for MVDS where the correlation was negative for CD3. See Tables 4.14 and 4.15 for details.

Table 4.20: Correlation of all the markers in the whole tissue sections with the vascular measurements

		MVDS	MVDF	MVD (SMC)	MVD (No SMC)	Wall thickness	Vessel distance	Fat cell diameter
vWF Total	**Correlation Coefficient**	-0.189	0.227	-0.020	-0.200	0.505	-0.176	-0.154
	P value	0.517	0.434	0.946	0.493	0.078	0.566	0.616
CD34 Total	**Correlation Coefficient**	-0.031	0.375	-0.110	-0.002	**0.538***	0.015	0.011
	P value	0.917	0.186	0.707	0.994	**0.047***	0.958	0.970
CD68 Total	**Correlation Coefficient**	0.002	**0.702****	**-0.698****	0.218	**0.714****	0.389	**0.569***
	P value	0.994	**0.005****	**0.005****	0.454	**0.004****	0.169	**0.034***
CD3 Total	**Correlation Coefficient**	-0.016	**0.613***	**-0.597***	0.135	**0.615***	0.346	**0.601***
	P value	0.957	**0.026***	**0.031***	0.661	**0.025***	0.247	**0.039***
CD20 Total	**Correlation Coefficient**	0.021	0.485	-0.049	0.098	0.545	0.056	0.489
	P value	0.948	0.110	0.880	0.762	0.067	0.863	0.09
Vim Total	**Correlation Coefficient**	0.027	**0.688****	**-0.801****	0.261	**0.741****	**0.547***	**0.653***
	P value	0.929	**0.009****	**0.001****	0.338	**0.002****	**0.043***	**0.011***

*Significant correlation at 0.05 ** Significant correlation at 0.01

4.6.7 Correlations in linear regression plots

The regression plots shown in Figures 4.25 to 4.31 explain the relationships of the different variables in a visual easy-to-follow way. For example, Figure 4.25 shows the relationship of age and BMI with vWF in synovium, fat and total sections. The positive correlation with age and the negative with BMI is shown very clearly. A similar pattern can be observed in Figure 4.26 for CD34 in the fat and total sections. In Figure, 4.26 mean fat cell diameter showed a significant negative correlation with age and a positive correlation with BMI.

The total measurements of CD3, CD20 and vimentin showed obvious negative correlations with age and positive correlations with BMI, as shown in Figure 4.27. The correlations between vascular measurements and age and BMI are shown in Figures 4.28 and 4.29. There was a positive correlation between age and the vessels with a smooth muscle layer whereas the opposite was observed with BMI. All the other vascular measurements correlated negatively with age and positively with BMI.

The CD68 correlations are shown in Figure 4.30. There is a negative correlation between age and CD68 in the synovium, fat and total sections. On the other hand, BMI correlated positively with CD68 measurements.

Figure 4.31 shows the correlations of CD3, CD20 and Vimentin in fat sections with age and BMI. They all exhibited a positive correlation with BMI and a negative correlation with age. Vimentin showed very significant correlations with age and BMI with a narrow confidence interval.

The overall correlations suggested an increased inflammatory response in the young age groups (*i.e.* groups 1 and 2). The increase in weight (*i.e.* group 1) also suggested an increase in the inflammatory response.
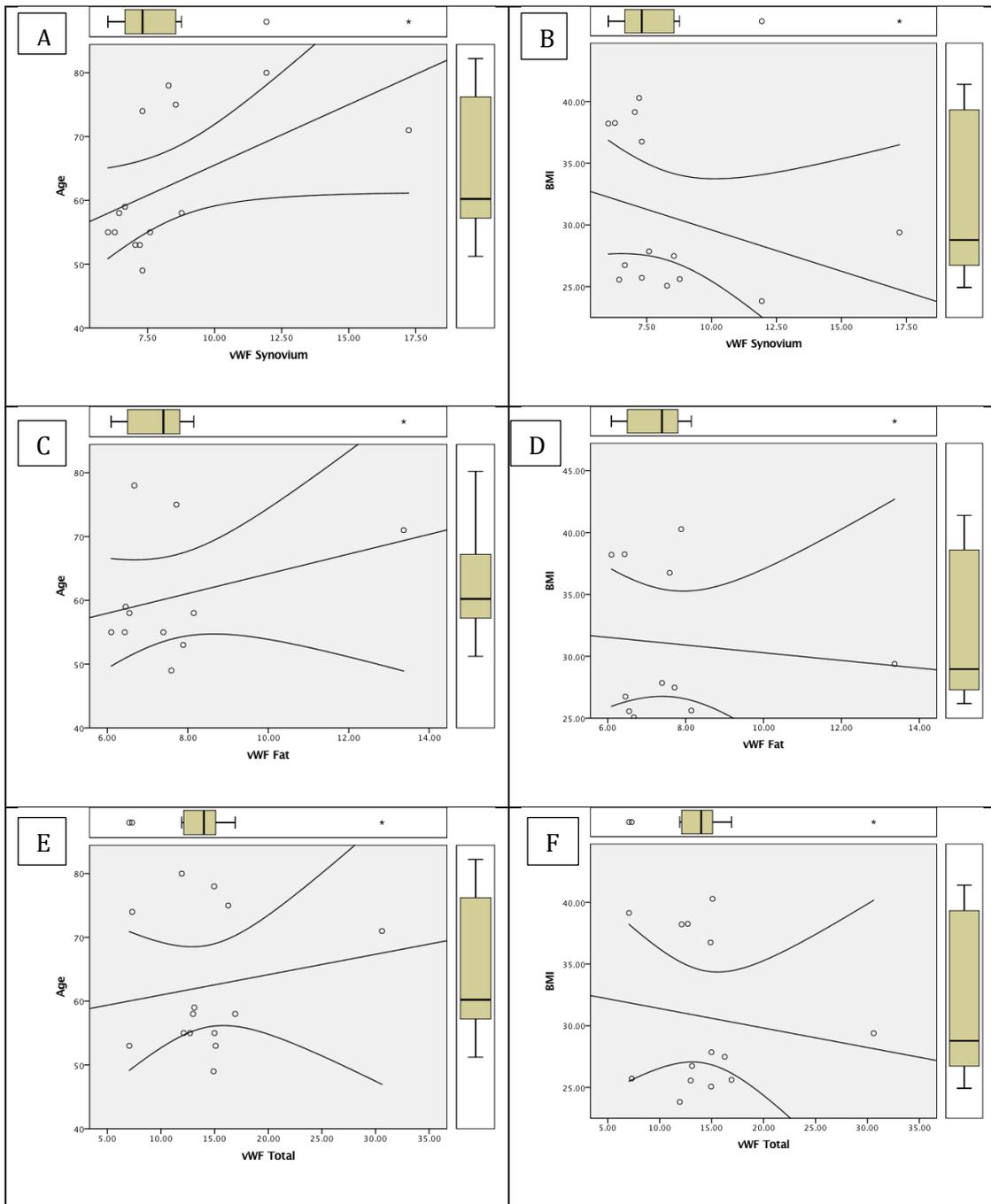
Figure 4.25: Regression plots, showing correlation of Age with vWF in the synovium (A), Fat(C) and vWF total (E). The correlation of BMI with vWF in the synovium (B), Fat (D) and vWF total (F). vWF showed Positive Correlation with the Age and negative correlation with BMI
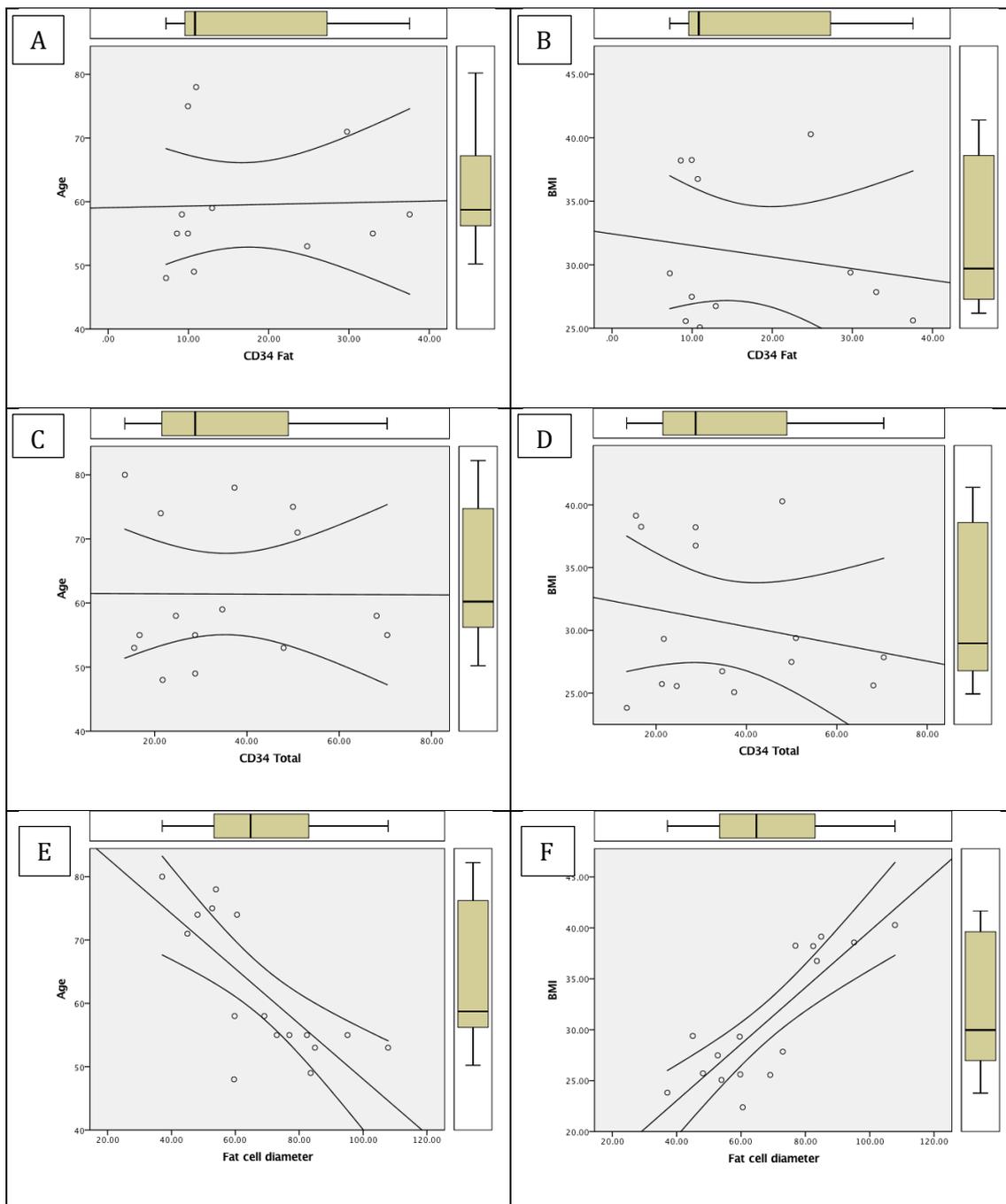
Figure 4.26: *Regression plots, showing correlation of Age with CD34 in fat (A), CD34 in total sections (C) and mean fat cells diameter (E). The correlation of BMI with CD34 in fat (A), CD34 in total sections (C) and mean fat cells diameter (E). CD34 showed very weak positive correlation with the age and negative correlation with BMI. Mean fat cells diameter showed significant negative correlation with age and significant positive correlation with BMI.*
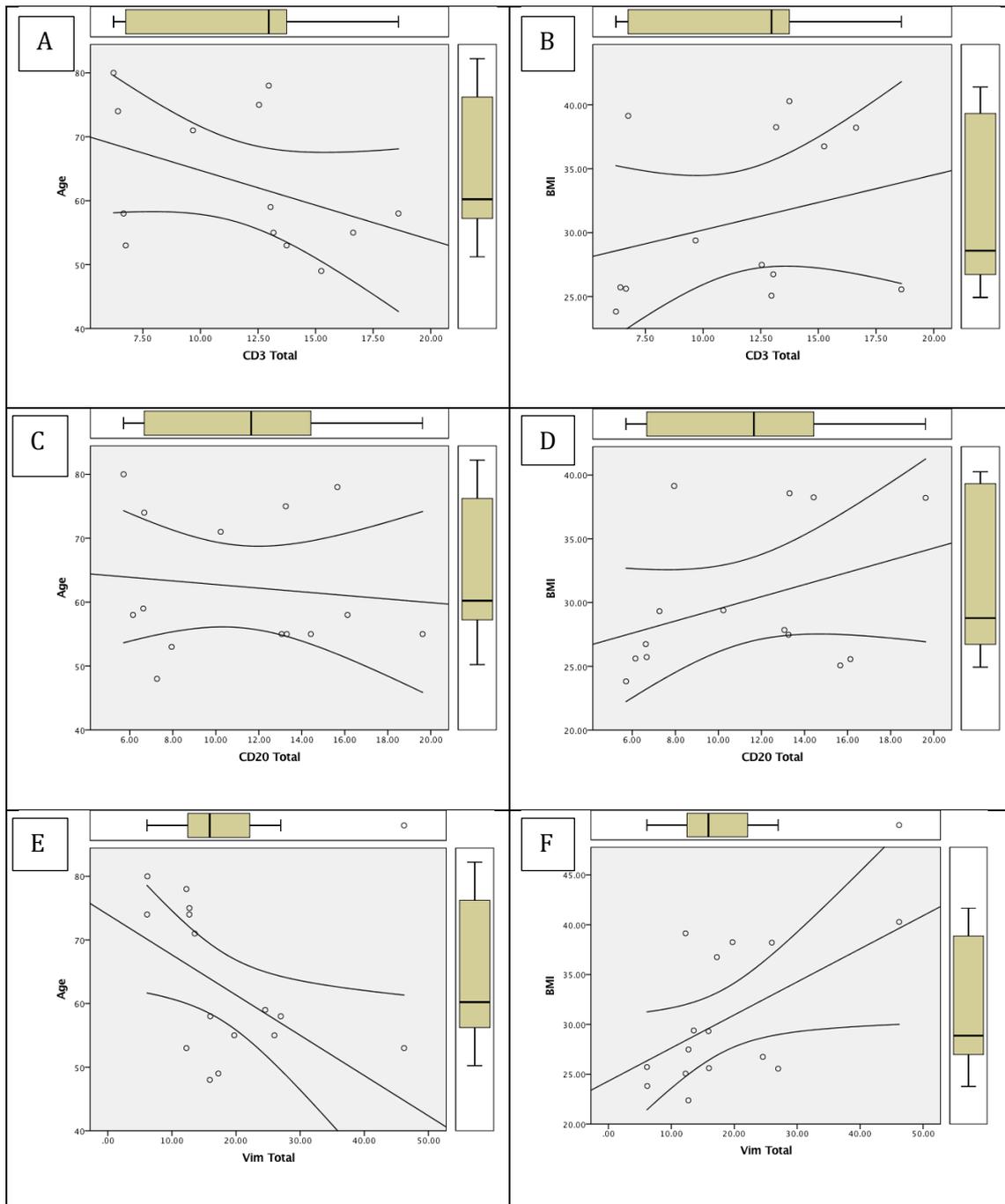
Figure 4.27: Regression plots, showing correlation of Age with CD3 total (A), CD20 total (C) and Vimentin total (E). The correlation of BMI with CD3 total (B), CD20 total (D) and Vimentin total (F). Age showed negative correlation with the above markers whereas BMI correlated positively.
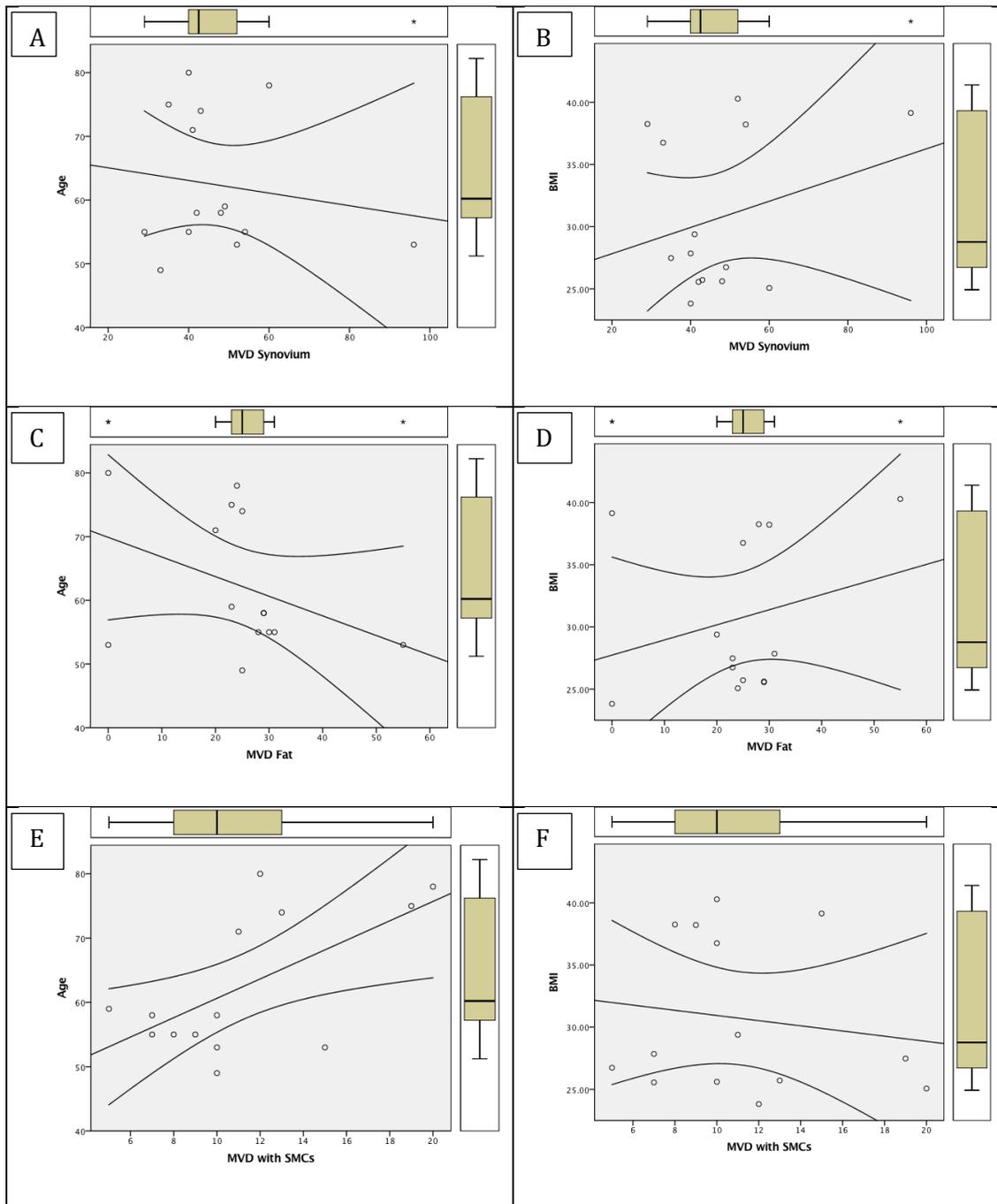
Figure 4.28*: Regression plots, showing correlation of Age with vascular density in the synovium (A), the vascular density in fat (C) and vessels with smooth muscle layer in the synovium (E). The correlation of BMI with vascular density in the synovium (B), the vascular density in fat (D) and vessels with smooth muscle layer in the synovium (F). Age showed negative correlation with the vascular density in synovium and fat sections whereas BMI correlated positively. Vessels with smooth muscle layer correlated positively with age and negatively with BMI.*
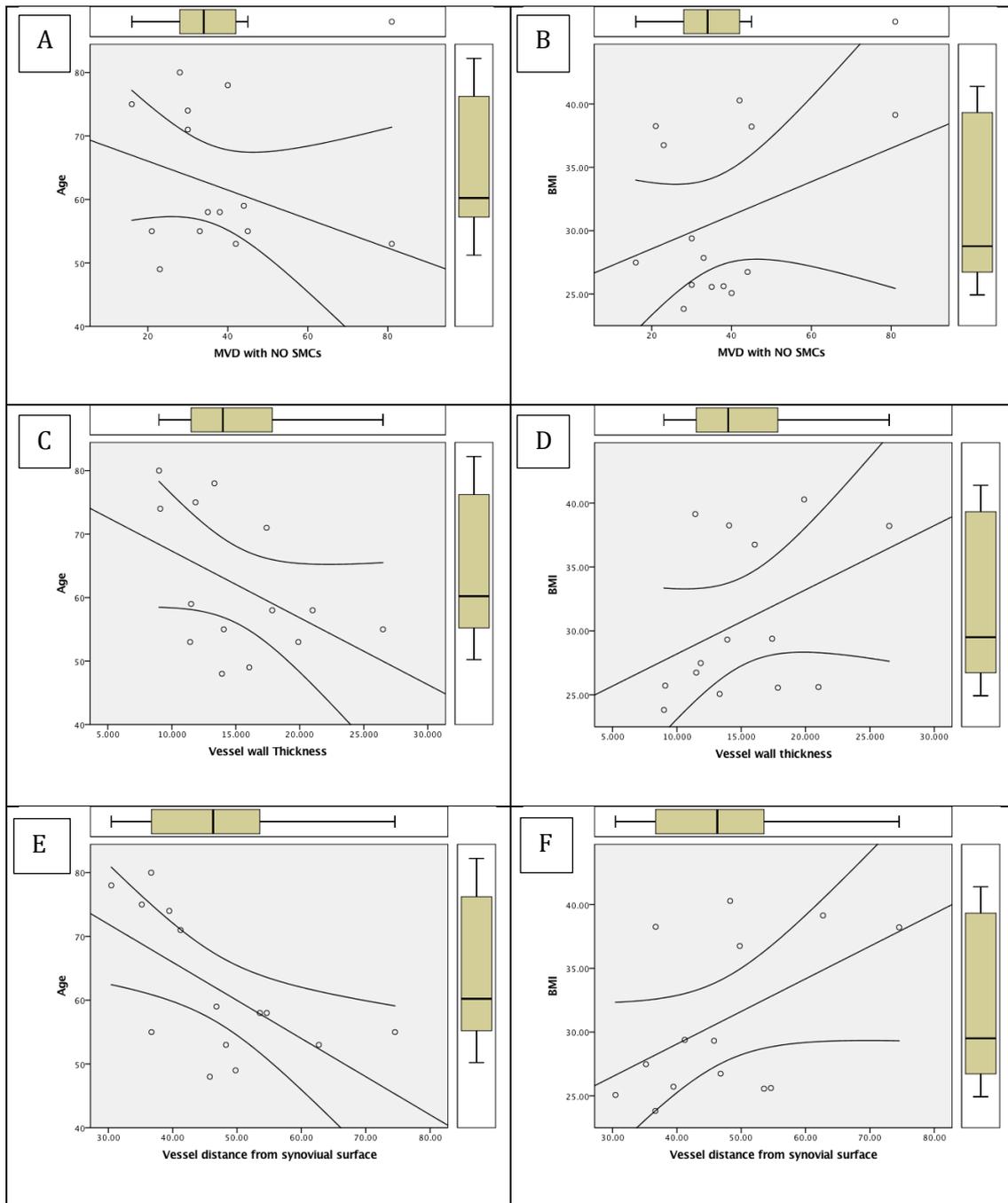
Figure 4.29: *Regression plots, showing correlation of Age with vessels lacking smooth muscle layer (A), mean vessel wall thickness (C) and mean intimal vessels distance from synovial surface (E). The correlation of BMI with vessels lacking smooth muscle layer (B), mean vessel wall thickness (D) and mean intimal vessels distance from synovial surface. Age showed negative correlation with the above measurements whereas BMI correlated positively.*
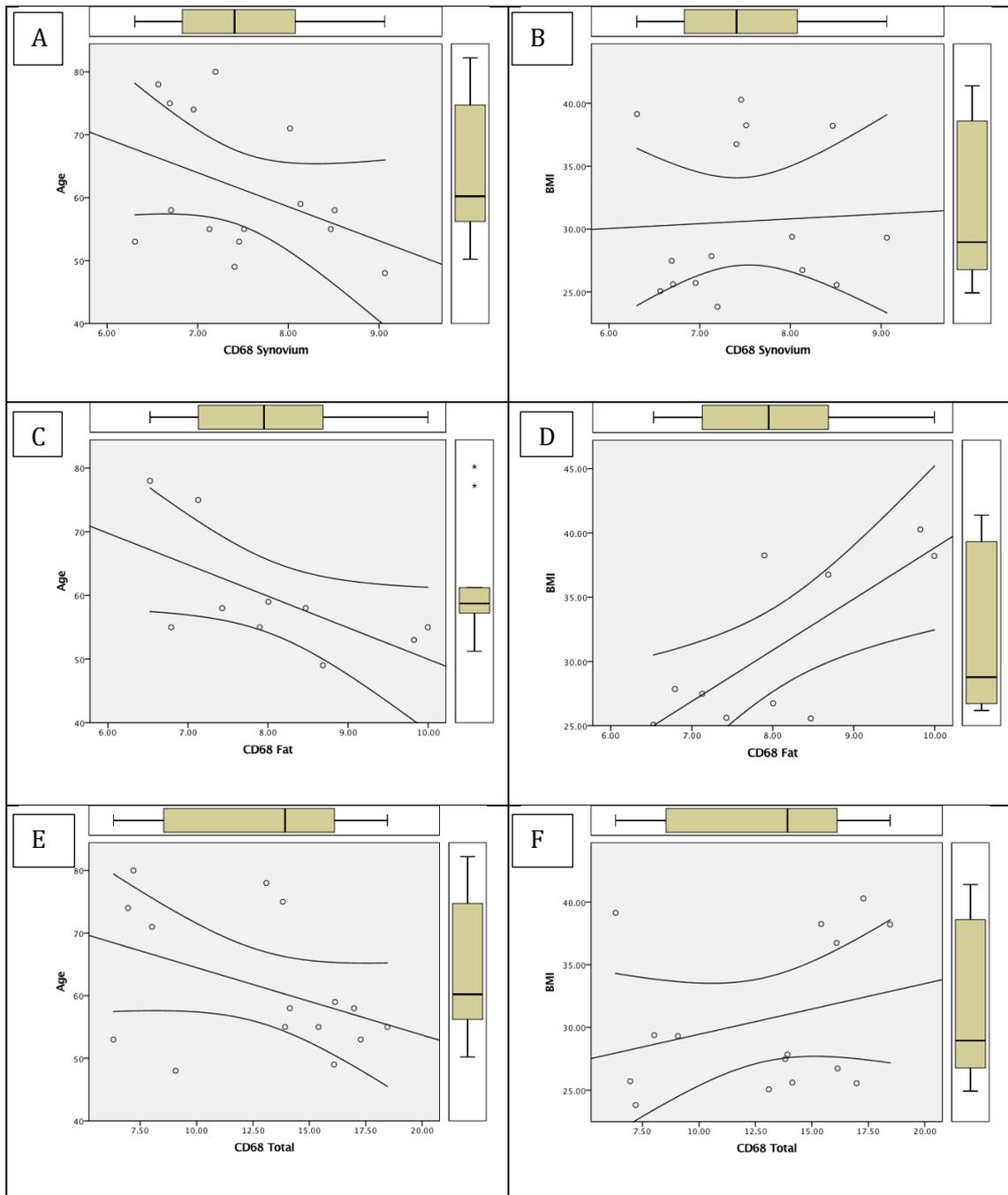
Figure 4.30: *Regression plots, showing correlation of age with CD68 in the synovial sections (A), CD68 in the fat sections(C) and CD68 in the total sections (E). The correlation of BMI with CD68 in the synovial sections (B), CD68 in the fat sections (D) and CD68 in the total sections (F). Age showed negative correlation with the above measurements whereas BMI correlated positively.*
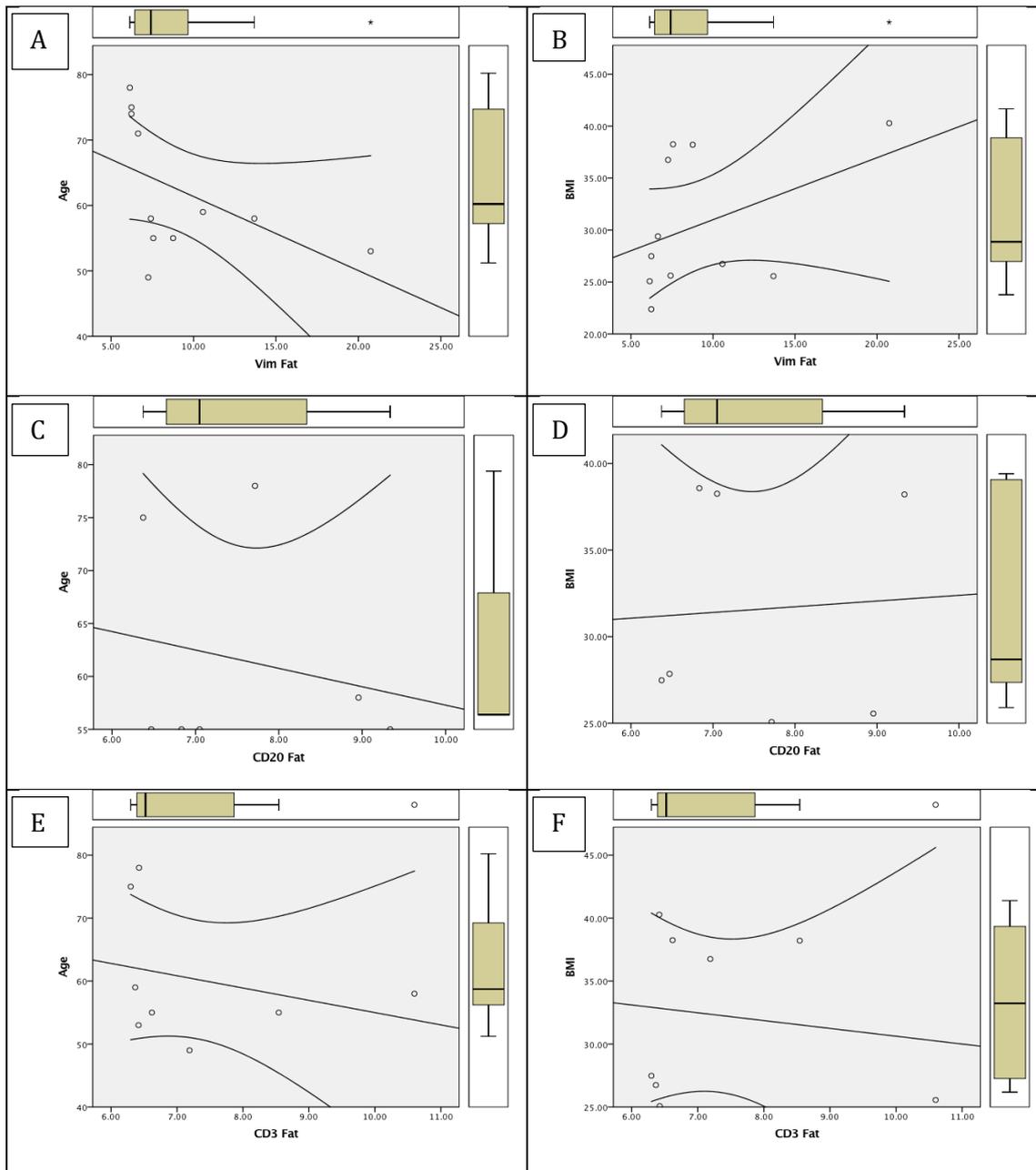
Figure 4.31: Regression plots, showing correlation of age with vimentin in fat sections (A), CD20 in the fat sections (C) and CD3 in the fat sections (E). The correlation of BMI with vimentin in fat sections (A), CD20 in the fat sections (C) and CD3 in the fat sections (E). Age showed negative correlation with the above measurements whereas BMI correlated positively.

4.6.8 Summary of correlations

A negative correlation was observed between age and CD68, CD3, CD20 and vimentin. In other words, the inflammatory response was lower in the older age

group. In contrast, there was a positive relationship with BMI. This indicates that an increase in BMI is associated with an increase in the inflammatory response. The vascular measurements showed a negative correlation with age and a positive one with BMI, except for the number of vessels with a smooth muscle layer.

4.7 Semi-quantitative assessments

The slides were also assessed using semi-quantitative scoring where the cells expression were assessed. Table 4.16 shows the results of the scoring.

4.7.1 vWF

vWF showed strong expression in the majority of the patients in terms of the stain intensity and the number of vessels. This does not agree with the ImageJ findings. However, this measures the intensity of expression rather than the actual surface area.

4.7.2 CD34

CD34 showed a very strong expression in all the patients, which explains the entire vascular expression in the whole sections. This follows the same pattern as vWF.

4.7.3 CD68

CD68 showed stronger expression in the young age group patients (groups 1 and group 2), whereas weaker expression was observed in the old age group people. This is in agreement with the ImageJ findings.

4.7.4 CD3

CD3 followed the same pattern as CD68 with more expression in groups 1 and 2 as compared to group 3. This also agreed with the ImageJ findings.

4.7.5 CD20

CD20 did not follow any specific trend. However, more expression was observed in groups 1 and 2.

4.7.6 Vimentin

Vimentin showed a very significant difference in terms of expression. It was strongly expressed in groups 1 and 2. On the other hand, the expression was weak in group 3. These findings match up with the ImageJ findings.

Table 4.21: Semi quantitative scores (G1: group 1, G2: group 2, G3: group 3; the number represent the number of patients in each category):

		0	1+	2+
vWF	G1	0	1	5
	G2	0	1	4
	G3	0	1	5
CD34	G1	0	0	6
	G2	0	0	5
	G3	0	0	6
CD68	G1	0	1	5
	G2	0	1	4
	G3	0	3	3
CD3	G1	0	3	3
	G2	1	1	3
	G3	1	3	2
CD20	G1	4	1	1
	G2	2	1	2
	G3	3	2	1
Vimentin	G1	0	0	6
	G2	0	0	5
	G3	0	6	0

4.8 Chronic synovitis scoring

The slides were assessed for evidence of synovitis. They were scored as shown in Table 4.17. There were no obvious differences among the groups. However, the majority of the patients in all three groups showed evidence of low-grade synovitis. The pie chart in Figure 4.32 shows the distribution of chronic synovitis among all the groups.

Table 4.22: Chronic synovitis scoring outcome.

	No synovitis	Low-grade synovitis	High-grade synovitis
Group 1	3	2	1
Group 2	1	4	0
Group 3	2	3	1
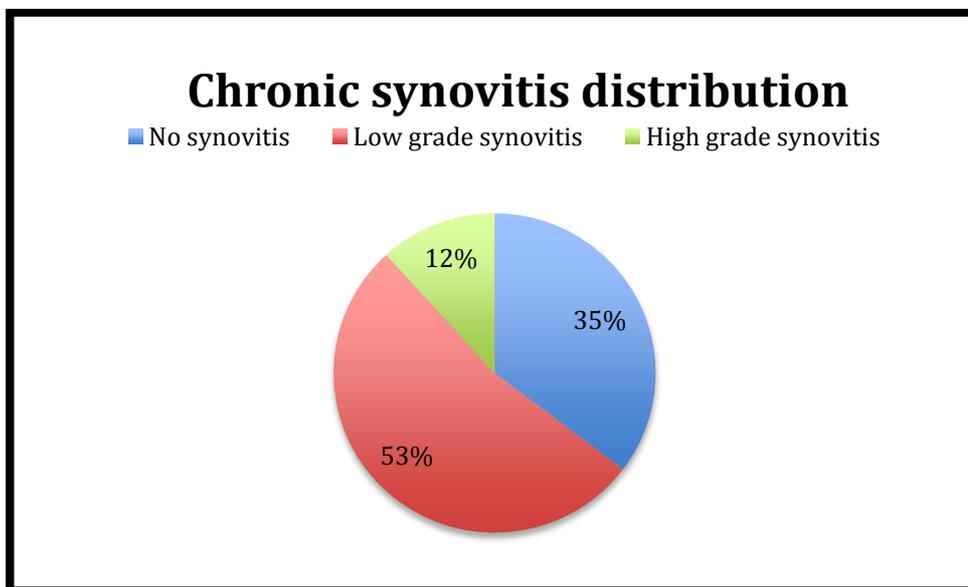
Figure 4.32: *Chronic synovitis distribution. Low grade synovitis is shown in over half of the patients.*

	Group 1	Group 2	Group3
vWF			
CD34			
CD68			
CD3			
CD20			
Vim			
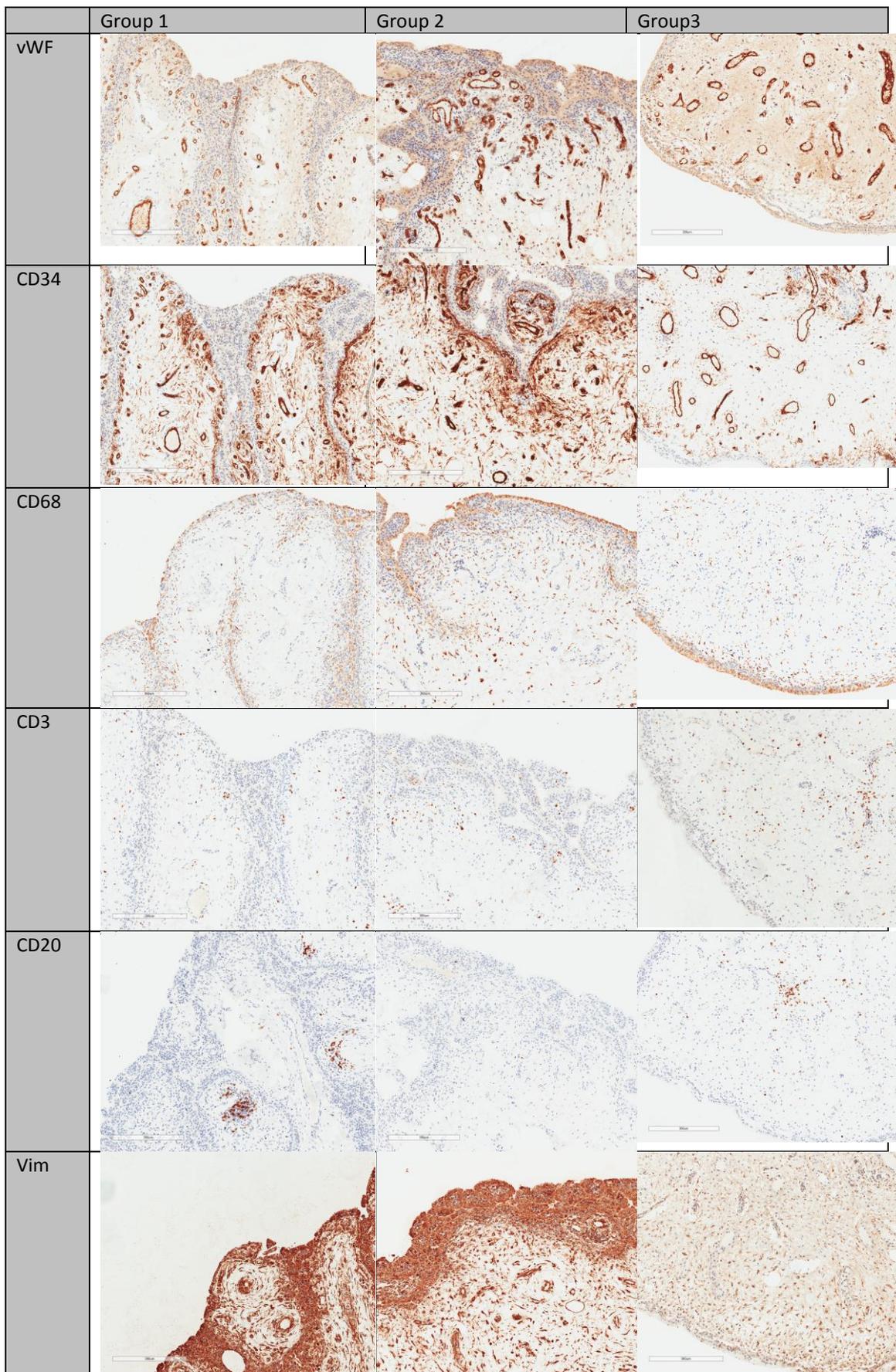
Figure 4. 33: *Examples of IHC staining of all the markers across groups*
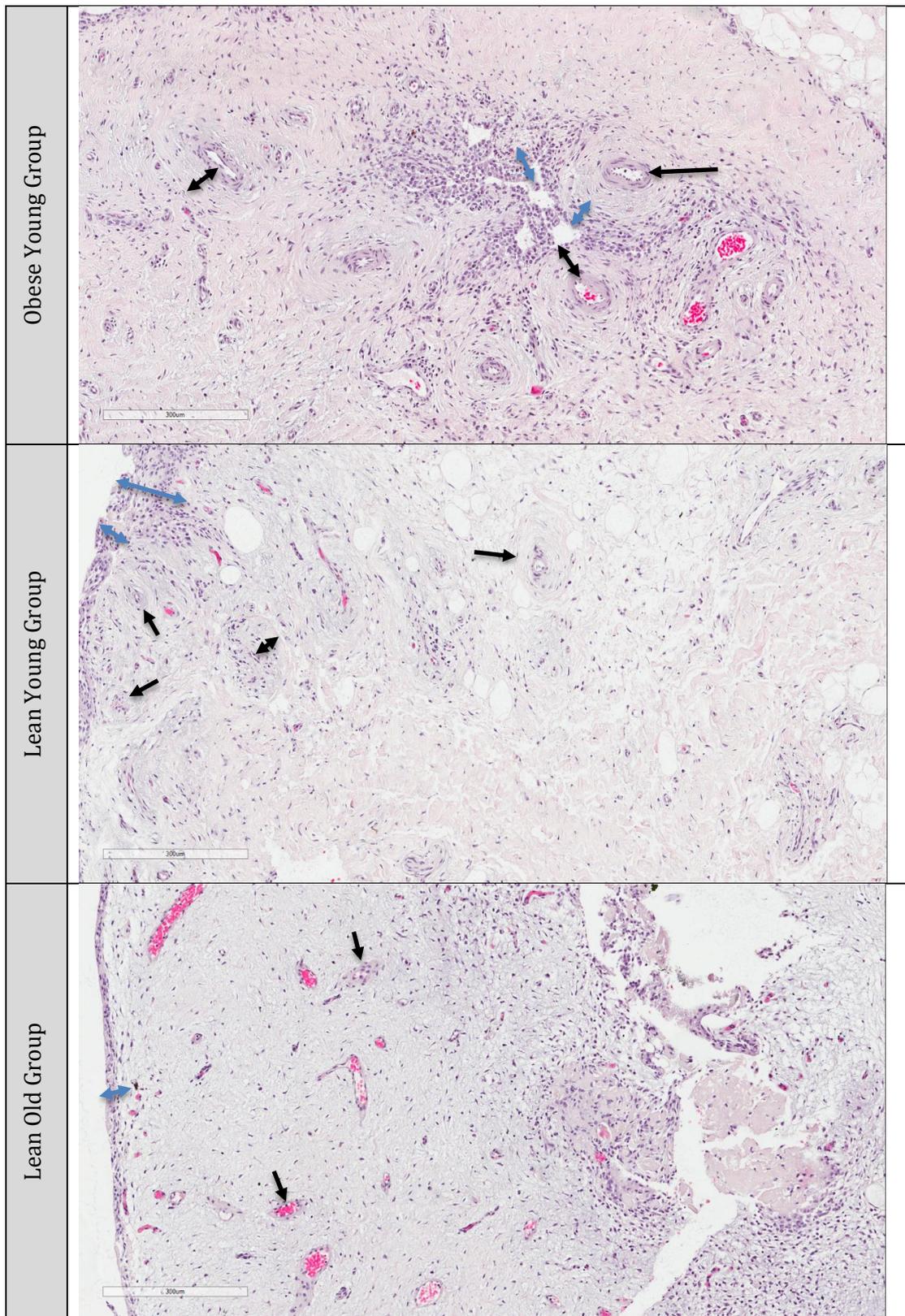
Figure 4. 34*: Examples of vascular wall thickness (the black single head arrows point to the vascular smooth muscle layers, the black double headed arrows point to the vessel wall thickness, the blue double headed arrows point to the synovial intima)*

4.9 Other abnormal findings

Vessels with intimal hyperplasia were observed in some sections, mainly in the young and more so in the young obese people (Figures 4.34 and 4.35).
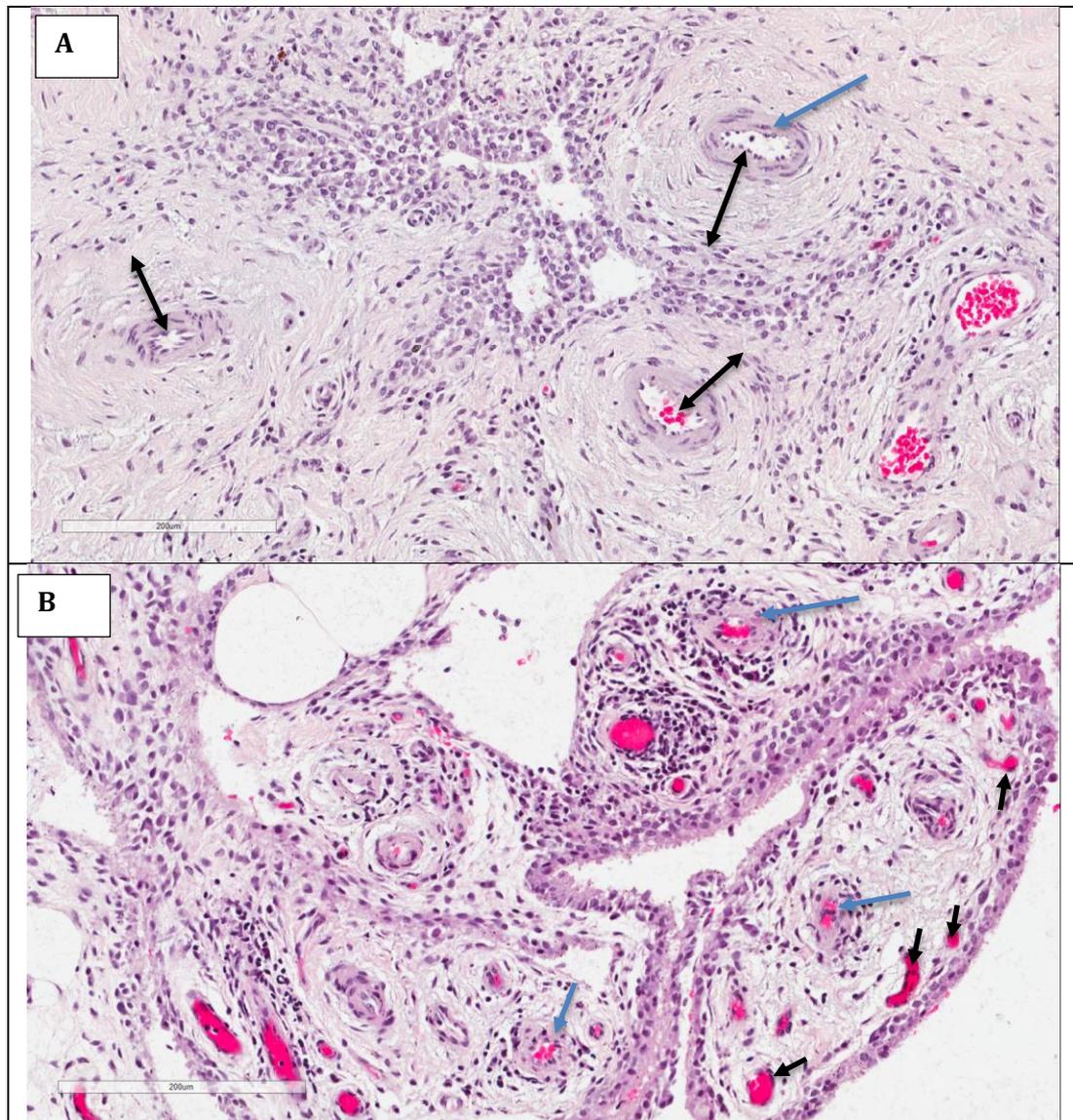
Figure 4. 35*: Synovial sections from obese people showing significant vascular hyperplasia very close to the intimal surface (blue arrows pointing to the smooth muscle layer, black double headed arrows point to the vascular wall thickness, single head small black arrows point to normal capillaries).*

The presences of the smooth muscle layers suggest that they are either arteries or arterioles. However, they are very close to the intimal synovial layer, so they are unlikely to be a bigger vessel like an artery. It is most likely that they represented

arterioles. It is expected to see capillaries at this level of the synovial sections to allow for the exchange of molecules. It is difficult to explain why these vessels have a smooth muscle layer very close to the intimal surface. One explanation is that it might suggest a local manifestation of a systemic problem like atherosclerosis, which is linked to obesity. However, this was also observed in the young lean people, the majority of whom are smokers.

5. DISCUSSION

5.1 Patient selection

OA is a multifactorial condition. Of the multiple risk factors, age and genetics have been established as independent risk factors (Goldring and Goldring, 2006). It is still believed that OA is a wear and tear problem. The understanding of OA has evolved with multiple theories existing to solve its mystery. There is growing evidence of a chronic inflammatory response involved in the process of OA development, especially metabolic syndrome (Brooks, 2003, Benito *et al.*, 2005, Smith *et al.*, 1997, Spector *et al.*, 1997, Martinek, 2003, Goldring and Otero, 2011, Clockaerts *et al.*, 2010). Despite the huge body of research on OA, there is nothing mentioned about the synovial changes in the young obese people in terms of inflammation and microcirculation. It is still a mysterious area. This project has focused on these two aspects of the synovium to gain in-depth knowledge of the changes encountered in this subgroup of patients. Moreover, young and old lean patients were studied in the same way to find out if there is a pattern of changes in their synovium. One of the questions that required an answer was why the old people manage to have their knee replacements very late in life compared to the young obese patients, who gets their knee replacements fairly early. What is it in their synovium that predisposes them to the early OA? Is it inflammation? Is it mechanical problems? Is it a local or systemic problem? What happens to their synovial microcirculation? All these questions require an answer.

To be able to answer these questions, careful patient selection was necessary. This type of research is difficult to carry out because of the nature of the condition and the multiple risk factors involved. The selection criteria were strict to avoid the influence of any risk factors that could affect the results. The groups of patients in this study were matched in many aspects. They all share the same degree of advanced OA as indicated by the radiological KL score and the functional WOMAC score. They all share similar levels of health status with almost similar ASA grades

and the number of comorbidities was evenly distributed among groups. Gender was also matched for male and female representation. However, smoking was more prevalent in the young lean group, which was not found to be significantly different when comparing smoking with all the markers. Moreover, to be able to illicit a difference as a result of smoking, a larger number of patients would be required. The selected group of patients differed in their age and BMI but were almost matched for other clinical risk factors.

5.2 The synovial microcirculation

Levick studied the microcirculation of the synovium in rabbits and humans and his work helped in the assessment of synovial microcirculation in this project (Levick, 1995). Others have also looked into the synovium and examined vascularity using vWF (Pessler *et al.*, 2008a, Pessler *et al.*, 2008b, Mucke *et al.*, 2016). In this project, a combination of methods was used to assess the vascular changes in relation to age and BMI. This is the first time that synovial vascularity has been assessed extensively. Several measurements were taken from the histological slides, including: digital measurement of stain surface area of vWF and CD34, manual count of intimal vascular density in the hot area, which is further divided to number of capillaries (no smooth muscle layer) and arterioles (smooth muscle layer present), the vascular wall thickness (hyperplasia) and the distance of the vessels to the surface of the synovium. The idea was to see if fat was associated with the vascular changes and how old people's synovia differ from those of young people.

The data of the overall vascularity using CD34 and the activated blood vessels using vWF showed an overall increasing trend of both markers in the old lean group. This finding might not be an accurate representation of the actual situation, as discussed in Section 3.3.1. Although the trend is obvious, there was no significant difference between groups in the overall vascularity. On the other hand, the manual counts of activated capillaries in the synovial intima showed a trend of increasing density in the young obese group. There were no previous papers to compare the microcirculation against.

Another parameter of importance is the vessel distance from the synovial surface, which is important to achieve easy transport of nutrients to the joint. A trend of increasing vessel distance from the synovial surface was noted in the young age groups. In fact, the vessel distance measurement of the old group people agreed with Levick's findings of normal vessel distance for the human synovium. Levick reported 35 µm for a normal synovium intimal vessel distance from the joint, which is very close to what was observed in this research (mean (SD) 36.66 µm (4.16)). This could be a reason why the old people maintain their articular surface for longer when compared to the young people with OA.

Vessel hyperplasia was also assessed as a local manifestation of the systemic problem of increased fat. There was no significant difference among the groups. However, a trend of increasing wall thickness was observed in the young obese people. More importantly, these changes were observed very close to the intimal surface of the synovium; an example of this finding is shown in Figure 4.35. One could argue that these vessels are actually larger vessels from the vascular tree. Although this might be true for sections deep in the synovium, it would not be accepted for intimal vessel to be that thick and be able to function. When combining the vessel wall thickness and the vessel distance from the synovial surface, obese people's joints will suffer from defective nutrient supply and hence early OA. Although this argument might be clear for the young obese people, it cannot explain why the young lean group suffers the same fate. It is possible that the smoking effect in this group caused these changes; however, this cannot be concluded as the condition is multifactorial and we do not know much about the detailed background of this groups in terms of activities and previous injuries that could have led to the advanced OA changes. Furthermore, the changes that are observed are more pronounced in the obese people compared to the other patients.

The groups were re-distributed based on the fat cell size and the measurements were reassessed to see if fat cell diameter (local fat burden) affects the vascularity. The data supported the previous findings. Increased fat cell size showed a significant

increase in the density of the vessels in the fat sections as well as the vessel distance to the synovial surface.

The semi-quantitative assessment did not show any differences across the groups for the vascular expressions. It could be argued that there is no significant difference in the overall vascularity because we are looking at a similar advanced stage of OA.

In summary, fat is definitely associated with changes to the synovial microvascularity, as observed in our data. It also may suggest a systemic problem, although this is difficult to prove until we examine other tissues.

5.3 Synovial inflammatory response

The inflammation in OA has been studied in the past and there are several articles that have addressed this before. However, it has not been looked at in relation to the fat cell diameter. The inflammatory markers used in this research were CD68 (macrophages), CD3 (T cells), CD20 (B cells) and vimentin (fibroblasts).

When looking into the comparison across groups, there were no significant differences noted, except for vimentin, which was significantly low in the old age group. Although there were no significant differences across groups, CD68, CD3 and CD20 showed an increasing trend toward the young groups with the highest levels in the young obese people.

The significant difference of vimentin observed in the old age group could be attributed to various reasons. Vimentin is a cytoskeleton protein that helps to maintain the structure of the cell (Fuchs and Weber, 1994). It is highly expressed in fibroblasts but also has some expression in other cells, including T and B cells. Fibroblasts are very well known for their role in tissue repair (Cheng *et al.*, 2016). This leads us to believe that the young people are experiencing ongoing tissue damage and repair, as expressed by the increased amount of vimentin expression in

the young age group. Another possibility is that the old age group do not cause tissue damage with their routine activity owing to their lower activity level. Furthermore, it could be that the tissue is not capable of producing more vimentin owing to age and cellular senescence.

However, when redistributing the patients into those with large and small fat cell diameters, there was a significant difference between groups. The large fat cells attracted more inflammatory cells. Thus, when ignoring age and BMI, the increased size of the fat cells was linked to an increased inflammatory response. These findings support the inflammatory theory of metabolic OA. This research finding is an agreement with the published data on the effect of fat on the low-grade inflammatory response (Clockaerts *et al.*, 2010, Goldring and Otero, 2011, Wang *et al.*, 2011, Nair *et al.*, 2012). Although there is very strong evidence that fat in obese people is an obvious cause of inflammation, the mechanical part of obesity cannot be ignored. Obese patients have greater sagittal-plane knee moments and greater knee-joint loads than normal-weight adults (Browning and Kram, 2007). Mechanical stress is important for the living of chondrocytes. However, injurious stress causes alteration of the articular cartilage extracellular matrix, leading to depletion of proteoglycan molecules and shedding of articular cartilage, which is considered to be inflammatory trigger on its own (Torzilli *et al.*, 2010, Ding *et al.*, 2010).

In summary, obesity acts through different mechanisms to cause early OA in young people. Fat makes a major contribution to the inflammatory response, as shown in our data, and it is expressed as a systemic problem, as demonstrated by the vascular changes.

5.4 The hypotheses

The main hypothesis is that vascular and inflammatory components of synovial tissue, debrided at primary knee arthroplasty for osteoarthritis, will vary with age and BMI. The increased fat leads to neo-angiogenesis and hence inflammation, which causes the early OA. Therefore, the null hypothesis has been rejected. There is

evidence of vascular and inflammatory differences among the groups of different ages and BMI.

The secondary questions were:

1- The effect of fat in the synovial tissue and its relation to angiogenesis. The data support adiposity as a cause of increased inflammation but not the only cause. In addition, the vascularity increased with increased fat cell diameter.

2- The changes in microcirculation in the synovium were studied and showed an interesting finding of variable wall thickness and distance from the synovial surface. The young obese patients showed an increase in both variables when compared to the other groups.

3- The relation of vascular changes and other inflammatory cells. The inflammatory cells were correlated positively with the vascular measurements, which could indicate that increased inflammation causes new vessel formation, which attracts more inflammatory cells.

4- The most predominant inflammatory cell type in all groups was macrophages. Fibroblasts were also observed in the young age groups (groups 1 and 2).

5- The synovitis scoring in all the groups was predominantly low grade. High-grade synovitis was also observed in all the groups but few patients had it.

5.5 Interesting finding

The synovium has been evaluated before but nothing has been mentioned about the vascular changes. A lot of attention was given to the cellular and extracellular changes. It was interesting to see very hyperplastic vessels very close to the synovial intimal surface. Levick gave a detailed description of what the vessel structure should look like to be able to carry out its function as a conduit of molecule exchange (Levick, 1995). More vascular hyperplasia was observed in the obese specimens and to a lesser extent in the other groups. One could argue that the vascular hyperplasia seen in the synovium could be the case in the rest of the body and this further supports the idea of the systemic metabolic process involved in obese people with OA. This was examined carefully to avoid misinterpretation. The

vascular anatomy changes depending on the section it is taken from. For example, the vessel could be taken at the level where it is still an artery and supposed to have a thick wall. Bearing this in mind, it could be that the sections have been taken at such a level. However, these vessels were observed very close to the intimal surface, where the vessels should be changing from arterioles to capillaries. These vessels most likely represent hyperplastic arterioles, indicating a change of atherosclerosis, which is a very well-established sign of a systemic vascular problem that is linked to obesity (Rocha and Libby, 2009, Libby *et al.*, 2010). This further supports the concept of a systemic problem of obesity and adiposity as a cause of early OA. The common link between all is inflammation.

We are led to believe that obesity and adiposity contribute to vascular dysfunction, which leads to a decrease in tissue nutrition. The tissue is placed under constant hypoxic drive, which causes the inflammation and early development of OA.

5.6 Study strength

Although this project is expected to have many limitations, it has some strengths. Its strength lies in the fact that it is the first time the synovium has been analysed in depth, especially for obese patients. A holistic approach to the synovium was followed starting from the gross appearances down to the details of the vascularity and the cellular changes involved in synovitis. The examination of the synovium was from the prospect of inflammation as a serious cause of early OA. Moreover, careful patient selection was done, which possibly led to the low sample size. The strict inclusion and exclusion criteria were important to avoid non-specific results, especially in a condition like OA where there are many factors that could lead to the same end result. Despite the huge diversity of patients from the age and BMI point of view, they were matched for the majority of other relevant clinical parameters. Furthermore, blinding was done while examining and analysing the ImageJ data, which was important to avoid bias in the adjustment of the threshold of stain detection.

5.6 Challenges and limitations

At the start of this project, it was anticipated that there would be many challenges owing to the nature of the condition. They include:

- The selection of patients to be grouped in different categories and then comparing them would raise the concern of comparing completely different groups and the comparison would then not be justified. However, this was considered and the groups were matched for almost all the parameters, except for age and BMI, which are the focus of this project and we wanted to see how they affect the condition.

- Although we tried to get all the patients within one group to be similar, each patient is different. They differ in terms of genetics, food, activities, previous injuries and medication. This might explain why there were some outliers, which were not consistent with any particular subject. These are non-modifiable risk factors.

- The disease nature of flare ups could affect the results. This is difficult to control.

- Sampling is another weak point. The tissues were not consistent in terms of content. The surgeon during the operation would take any tissue and hand it to the research team. The surgeon could be biased in terms of what tissue to provide. These sample were already available prior to the start of the project and could not be altered.

- Synovitis is not necessarily the same throughout the joint. There will be areas of florid synovitis and others with less inflammation within the same joint. This will definitely affect the results.

- Steroid injection could affect the findings but all the patients had steroid injections prior to their surgery and this factor was matched.

- Smoking could not be avoided and its effect could not be ruled out in the young lean subjects. It was difficult to exclude the smokers as the sample size was too small.

- Although blinding was done for the ImageJ analysis, it was not possible to do it for the other measurements as the investigator had to examine the whole section for every single patient to get the best representative sections. Furthermore, there was a very small number of patients and the slides were very easy to remember from the beginning, which precluded the blinding.

- Although all the data supported each other in the quantitative and semi-quantitative assessment, bias in the examination of the samples cannot be excluded.

- There was no pathologist support in this research project. The pathologist could have helped in tissue examination and selection of the representative sections for analysis. This was difficult to organise owing to the busy nature of the pathologist with interest in the synovium within the University of Liverpool.

- This project is trying to relate a very minute tissue sample to the patient and it is difficult to draw major conclusions.

- Although IHC is a well-established, largely accepted technique in both clinical and experimental parts of medical research, it has some limitations. The results vary for technical reasons, which include a lack of standard methodology and quality control between laboratories, especially with regard to antigen retrieval and type and degree of fixation. Moreover, it is expensive and time-consuming, and for these reasons it was not possible to test for more antibodies that could have helped in this project.

- Finally, the sample size is too small to be able to draw a strong conclusion. However, sample size calculation was done prior to patient recruitment.

5.7 Future work

It is important to tackle all the challenges outlined in this project to reach more robust conclusions. Suggestions for future work include:

- Follow the same recruitment criteria to get more patients and increase the sample size.

- Involvement of the pathologist from the start.

- Have a standard approach to sample collection. I would suggest getting three samples from the joint, namely: medial gutter, lateral gutter and supra-patellar pouch. The three samples should be fixed in the paraffin blocks for investigation separately and this could provide more detail of how the synovitis is expressed in the synovium. In addition, provide detailed intra-operative findings with a photograph of the joint. This will give a better perspective of what is actually happening. This way we can relate the small tissue sample to the patient.

- Blinding of the investigator to the samples is very important and will add a lot of strength to the study.

- Vascular anatomy and assessment of smooth muscle cells. This could be done using markers of synthesis and contractile phenotype of smooth muscle cells.

- It would be interesting to see the type of macrophage in the synovium and relate it to the circulating macrophages in the plasma.

6. CONCLUSION

OA is a complex, multifactorial pathology. It is the nature of the disease that makes it very difficult to identify the cause of early OA in obese people. Every patient is different from the others, even within the same group. These factors have been discussed in the challenges section (section 5.6). Despite the difficulties that have been encountered in this research project and the small number of samples, several conclusions can be made from this research, which can be summarised as follows:

1- OA is a complex multifactorial pathology with multiple confounding factors that could affect the results of any study.

2- Vascular density was not significantly different among all patients.

3- Vascular wall thickness and distance from the joint were greater in the young age groups and highest in the young obese group.

4- Vascular hyperplasia may suggest atherosclerosis, which is an indication of a systemic manifestation of adiposity.

5- The vascular dysfunction leads to failure of tissue nutrition, which contributes to the inflammatory response owing to the constant hypoxic drive, which causes the inflammation and early development of OA.

6- The old age group exhibited a lower inflammatory response compared to the younger group.

7- Obese patients showed the highest inflammatory response among all the groups.

8- Increased fat cell diameter was associated with more inflammatory cells and was associated with vascular changes (increase in vascular wall thickness and density in the fatty sections).

7. APPENDICES:

7.1 Appendix: 1

Orthopaedic outpatient clinics (NHS)

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Assessed for eligibility

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Inclusion criteria:
- Patient is willing and able to give informed consent for participation in the study
- Age < 60 with BMI >30
- Age <60 with BMI <30
- Aged >70 with BMI < 30
- Diagnosed with idiopathic OA
- Undergoing primary arthroplasty

Excluded: those screened who do not meet the inclusion criteria or do meet the exclusion criteria

↓

Informed Consent

↓

Baseline data collection Demographics BMI

↓

Surgery: Primary Arthroplasty

↓

| <60 (BMI >30) | <60 (BMI <30) | >70 (BMI <30) |

↓

Histology and IHC study

↓

Collect data and analyse

7.2   Appendix: 2

Heat-induced Epitope Retrieval Using the Dako PT-Link & Immunohistochemistry – use of the DAKO autostainer

Introduction:

The DAKO Autostainer is an automated slide processing system designed to automate manual staining methods routinely used in immunocytochemistry.

Each staining run can process 1 to 48 slides. Slides are secured in a horizontal position and reagents are dispensed onto the tissue by use of Teflon-coated pipettes. Incubations proceed at room temperature and individual slides can be programmed to receive different reagents.   The system provides intuitive programming routines, reagent and slide loading maps and Start/Finish run time displays.

Heat-induced Epitope Retrieval Using the Dako PT-Link

1. The PT-Link was turned on to begin heating to 65°C to begin (approx 20 minutes).
2. Slides were labelled with antibody, dilution and date, using a pencil.
3. Once 65°C is reached, the slides were placed in a staining rack (frosted end towards the top), which was placed in the PT-Link (in high PH medium).
4. The lid was closed and the heated started (The PT Link will heat to 96°C, hold for 20 minutes, and return to 65°C (approx 1 hour 10 minutes)
5. After PT-Link, the slides were washed with Envision Flex-Wash buffer for **5-15 minutes**.
6. The slides were not allowed to get dry throughout the staining procedure.

1. **Marking up & preparation of slides for the DAKO Autostainer**

   - Slides prepared
   - PT Link retrievals was done

2. **Preparing and programming the DAKO Autostainer**

   - Hazardous waste container was checked to ensure enough capacity

to accommodate waste from staining run.

- Ensured the levels of 1x FLEX wash buffer and de-ionized water in containers were sufficient for run requirements.
- All reagents required for staining run were brought to room temperature.
- The computer was programmed according to the standard steps of IHC staining.
- Once all the antibodies and all other reagents have been prepared, they were loaded in the machine.
- Following antigen retrieval, the slides were soaked in a rinse station containing Envision™ FLEX Wash Buffer for 5 mins prior to loading onto the Autostainer.
- The slides were correctly loaded into the slide racks according to the programmed map.
- The autostainer lid was closed and the Start Run sequence was initiated.
- The run included:
  - Envision-Flex block incubatation at room temperature for **5 minutes**.
  - Incubate primary antibody after dilution with appropriate concentration using antibody diluent (Envision-Flex) for **30 minutes**.
  - Appropriate linker (i.e. mouse for mouse primary antibody) was added and incubated at room temperature for **15 minutes**.
  - HRP was added and incubated at room temperature for **20 minutes**.
  - DAB was finally added and incubated at room temperature for **20 minutes**.
  - Transfer slides to horse-shaped rack.
  - The slides were unloaded and placed into rack in bath of dH$_2$O
- The autostainer was turned off after the complete run.

3. **Counterstaining and mounting the slides**
- Counterstain with Haematoxylin solution for 1 min was done
- Then the slides were Dipped in acid alcohol and rinsed in tap water
- Then Dipped in ammonia water for 30 sec and rinsed in tap water before returning slides to bath of dH$_2$O.
- The slides were dehydrated through five changes of IMS (Industrial Methylated Spirits) and 2x xylene in the fume hood.
- Finally, the slides were mounted in DPX.

7.3   Appendix: 3

SF-12 (Short-Form)

<u>**Question 1**</u>

In general, would you say your health is excellent, very good, good, fair, or poor?

○ Excellent   ○ Very Good   ○ Good   ○ Fair   ○ Poor

The following items are about activities you might do during a typical day.

<u>**Question 2**</u>

Does your health now limit you in these activities? If so, how much? First, moderate activities such as moving a table, pushing a vacuum cleaner, bowling or playing golf. Does your health now limit you a lot, limit you a little, or not limit you at all.

○ Limited a lot   ○ Limited a little   ○ Not limited at all

<u>**Question 3**</u>

Climbing several flights of stairs. Does your health now limit you a lot, limit you a little, or not limit you at all?

○ Limited a lot   ○ Limited a little   ○ Not limited at all

<u>**Question 4**</u>

During the past four weeks, have you accomplished less than you would like as a result of your physical health?

○ No   ○ Yes

<u>**Question 5**</u>

During the past four weeks, were you limited in the kind of work or other regular activities you do as a result of your physical health?

○ No   ○ Yes

<u>**Question 6**</u>

During the past four weeks, have you accomplished less than you would like to as a result of any emotional problems, such as feeling depressed or anxious?

○ No   ○ Yes

<u>**Question 7**</u>

During the past four weeks, did you not do work or other regular activities as carefully as usual as a result of any emotional problems such as feeling depressed or anxious?

○ No   ○ Yes

KNEE

Question 8

During the past four weeks, how much did pain interfere with your normal work, including both work outside the home and housework? Did it interfere not at all, slightly, moderately, quite a bit, or extremely?

○ Not at all   ○ Slightly   ○ Moderately   ○ Quite a bit   ○ Extremely

These questions are about how you feel and how things have been with you during the past 4 weeks. For each question, please give the one answer that comes closest to the way you have been feeling.

Question 9

How much time during the past 4 weeks have you felt calm and peaceful? All of the time, most of the time, a good bit of the time, some of the time, a little of the time, or none of the time?

○ All of the time   ○ Most of the time   ○ A good bit of the time

○ Some of the time   ○ A little of the time   ○ None of the time

Question 10

How much of the time during the past 4 weeks did you have a lot of energy? All of the time, most of the time, a good bit of the time, some of the time, a little of the time, or none of the time?

○ All of the time   ○ Most of the time   ○ A good bit of the time

○ Some of the time   ○ A little of the time   ○ None of the time

Question 11

How much time during the past 4 weeks have you felt down? All of the time, most of the time, a good bit of the time, some of the time, a little of the time, or none of the time?

○ All of the time   ○ Most of the time   ○ A good bit of the time

○ Some of the time   ○ A little of the time   ○ None of the time

Question 12

During the past 4 weeks, how much of the time has your physical health or emotional problems interfered with your social activities like visiting with friends, relatives etc? All of the time, most of the time, some of the time, a little of the time, or none of the time?

○ All of the time   ○ Most of the time   ○ Some of the time

○ A little of the time   ○ None of the time

Total Score:

KNEE

7.4   Appendix: 4

WOMAC Knee Score

INSTRUCTIONS: This survey asks for your view about your knee. This information will help us keep track of how you feel about your knee and how well you are able to do your usual activities.

Answer every question by ticking the appropriate box. If you are unsure about how to answer a question, please give the best answer you can.

Symptoms - These questions should be answered thinking of your knee symptoms during the last week.

S1. Do you have swelling in your knee?

Never	Often	Rarely	Sometimes	Always

S2. Do you feel grinding, hear clicking or any other type of noise when your knee moves?

Never	Often	Rarely	Sometimes	Always

S3. Does your knee catch or hang up when moving?

Never	Often	Rarely	Sometimes	Always

S4. Can you straighten your knee fully?

Never	Often	Rarely	Sometimes	Always

S5. Can you bend your knee fully?

Never	Often	Rarely	Sometimes	Always

Stiffness - The following questions concern the amount of joint stiffness you have experienced during the last week in your knee. Stiffness is a sensation of restriction or slowness in the ease with which you move your knee joint.

S6. How severe is your knee joint stiffness after first wakening in the morning?

None	Mild	Moderate	Severe	Extreme

S7. How severe is your knee stiffness after sitting, lying or resting later in the day?

None	Mild	Moderate	Severe	Extreme

Pain1

P1. How often do you experience knee pain?

Never	Monthly	Weekly	Daily	Always

KNEE

What amount of knee pain have you experienced the last week during the following activities?

P2. Twisting/pivoting on your knee

None	Mild	Moderate	Severe	Extreme

P3. Straightening knee fully

None	Mild	Moderate	Severe	Extreme

P4. Bending knee fully

None	Mild	Moderate	Severe	Extreme

P5. Walking on flat surface

None	Mild	Moderate	Severe	Extreme

P6. Going up or down stairs

None	Mild	Moderate	Severe	Extreme

P7. At night while in bed

None	Mild	Moderate	Severe	Extreme

P8. Sitting or lying

None	Mild	Moderate	Severe	Extreme

P9. Standing upright

None	Mild	Moderate	Severe	Extreme

Function, daily living - The following questions concern your physical function. By this we mean your ability to move around and to look after yourself. For each of the following activities please indicate the degree of difficulty you have experienced in the last week due to your knee.

Al. Descending stairs

None	Mild	Moderate	Severe	Extreme

A2. Ascending stairs

None	Mild	Moderate	Severe	Extreme

KNEE

For each of the following activities please indicate the degree of difficulty you have experienced in the last week due to your knee.

A3. Rising from sitting

None	Mild	Moderate	Severe	Extreme

A4. Standing

None	Mild	Moderate	Severe	Extreme

A5. Bending to floor/pick up an object

None	Mild	Moderate	Severe	Extreme

A6. Walking on flat surface

None	Mild	Moderate	Severe	Extreme

A7. Getting in/out of car

None	Mild	Moderate	Severe	Extreme

A8. Going shopping

None	Mild	Moderate	Severe	Extreme

A9. Putting on socks/stockings

None	Mild	Moderate	Severe	Extreme

A10. Rising from bed

None	Mild	Moderate	Severe	Extreme

A11. Taking off socks/stockings

None	Mild	Moderate	Severe	Extreme

A12. Lying in bed (turning over, maintaining knee position)

None	Mild	Moderate	Severe	Extreme

A13. Getting in/out of bath

None	Mild	Moderate	Severe	Extreme

A14. Sitting

KNEE

None	Mild	Moderate	Severe	Extreme

A15. Getting on/off toilet

None	Mild	Moderate	Severe	Extreme

For each of the following activities please indicate the degree of difficulty you have experienced in the last week due to your knee

A16. Heavy domestic duties (moving heavy boxes, scrubbing floors, etc)

Never	Rarely	Sometimes	Often	Always

A17. Light domestic duties (cooking, dusting, etc)

Never	Rarely	Sometimes	Often	Always

Thank you very much for completing all the questions in this questionnaire.

Notes:

Total Score:

KNEE

8. REFERENCES:

Alberts B, Johnson A, Lewis J, et al. Molecular Biology of the Cell. 4th edition. New York: Garland Science; 2002. Available from: https://www.ncbi.nlm.nih.gov/books/NBK21054/

ASKARY, A., SMEETON, J., PAUL, S., SCHINDLER, S., BRAASCH, I., ELLIS, N. A.POSTLETHWAIT, J., MILLER, C. T. & CRUMP, J. G. 2016. Ancient origin of lubricated joints in bony vertebrates. *Elife,* 5.

ATTUR, M., BELITSKAYA-LEVY, I., OH, C., KRASNOKUTSKY, S., GREENBERG, J., SAMUELS, J., SMILES, S., LEE, S., PATEL, J., AL-MUSSAWIR, H., MCDANIEL, G., KRAUS, V. B. & ABRAMSON, S. B. 2011. Increased interleukin-1beta gene expression in peripheral blood leukocytes is associated with increased pain and predicts risk for

AVIVI, I., STROOPINSKY, D. & KATZ, T. 2013. Anti-CD20 monoclonal antibodies: beyond B-cells. *Blood Rev,* 27**,** 217-23.

BABICH, V., MELI, A., KNIPE, L., DEMPSTER, J. E., SKEHEL, P., HANNAH, M. J. & CARTER, T. 2008. Selective release of molecules from Weibel-Palade bodies during a lingering kiss. *Blood,* 111**,** 5282-90.

BANCROFT JD, G. M. 2008. *Theory and practice of histological techniques. ,* Philadelphia Elsevier.

BARLAND, P., NOVIKOFF, A. B. & HAMERMAN, D. 1962. Electron microscopy of the human synovial membrane. *J Cell Biol,* 14**,** 207-20.

BEMIS, W. E. 1986. Feeding systems of living dipnoi: anatomy and function. *Journal of Morphology. ,* 249–275. .

BENITO, M. J., VEALE, D. J., FITZGERALD, O., VAN DEN BERG, W. B. & BRESNIHAN, B. 2005. Synovial tissue inflammation in early and late osteoarthritis. *Ann Rheum Dis,* 64**,** 1263-7.

BERENBAUM, F. 2013. Osteoarthritis as an inflammatory disease (osteoarthritis is not osteoarthrosis!). *Osteoarthritis Cartilage,* 21**,** 16-21.

BLAGOJEVIC, M., JINKS, C., JEFFERY, A. & JORDAN, K. P. 2010. Risk factors for onset of osteoarthritis of the knee in older adults: a systematic review and meta-analysis. *Osteoarthritis Cartilage,* 18**,** 24-33.

BOLTJES, A. & VAN WIJK, F. 2014. Human dendritic cell functional specialization in steady-state and inflammation. *Front Immunol,* 5**,** 131.

BOROSS, P. & LEUSEN, J. H. 2012. Mechanisms of action of CD20 antibodies. *Am J Cancer Res,* 2**,** 676-90.

BROOKS, P. 2003. Inflammation as an important feature of osteoarthritis. *Bull World Health Organ,* 81**,** 689-90.

BROWNING, R. C. & KRAM, R. 2007. Effects of obesity on the biomechanics of walking at different speeds. *Med Sci Sports Exerc,* 39**,** 1632-41.

CABECADAS, J. M. & ISAACSON, P. G. 1991. Phenotyping of T-cell lymphomas in paraffin sections--which antibodies? *Histopathology,* 19**,** 419-24.

CARMAN, W. J., SOWERS, M., HAWTHORNE, V. M. & WEISSFELD, L. A. 1994. Obesity as a risk factor for osteoarthritis of the hand and wrist: a prospective study. *Am J Epidemiol,* 139**,** 119-29.

CHARO, I. F. & RANSOHOFF, R. M. 2006. The many roles of chemokines and chemokine receptors in inflammation. *N Engl J Med,* 354**,** 610-21.

CHARO, I. F. & TAUBMAN, M. B. 2004. Chemokines in the pathogenesis of vascular disease. *Circ Res,* 95**,** 858-66.

CHENG, F., SHEN, Y., MOHANASUNDARAM, P., LINDSTROM, M., IVASKA, J., NY, T. & ERIKSSON, J. E. 2016. Vimentin coordinates fibroblast proliferation and keratinocyte differentiation in wound healing via TGF-beta-Slug signaling. *Proc Natl Acad Sci U S A,* 113**,** E4320-7.

CHETTY, R. & GATTER, K. 1994. CD3: structure, function, and role of immunostaining in clinical practice. *J Pathol,* 173**,** 303-7.

CHIDLOW, J. H., JR., SHUKLA, D., GRISHAM, M. B. & KEVIL, C. G. 2007. Pathogenic angiogenesis in IBD and experimental colitis: new ideas and therapeutic avenues. *Am J Physiol Gastrointest Liver Physiol,* 293**,** G5-g18.

CHIOLERO, A., FAEH, D., PACCAUD, F. & CORNUZ, J. 2008. Consequences of smoking for body weight, body fat distribution, and insulin resistance. *Am J Clin Nutr,* 87**,** 801-9.

CHOY, E. H. & PANAYI, G. S. 2001. Cytokine pathways and joint inflammation in rheumatoid arthritis. *N Engl J Med,* 344**,** 907-16.

CHRISTENSEN, R., BARTELS, E. M., ASTRUP, A. & BLIDDAL, H. 2007. Effect of weight reduction in obese patients diagnosed with knee osteoarthritis: a systematic review and meta-analysis. *Ann Rheum Dis,* 66**,** 433-9.

CIVIN, C. I., STRAUSS, L. C., BROVALL, C., FACKLER, M. J., SCHWARTZ, J. F. & SHAPER, J. H. 1984. Antigenic analysis of hematopoiesis. III. A hematopoietic progenitor cell surface antigen defined by a monoclonal antibody raised against KG-1a cells. *J Immunol,* 133**,** 157-65.

CLOCKAERTS, S., BASTIAANSEN-JENNISKENS, Y. M., RUNHAAR, J., VAN OSCH, G. J., VAN OFFEL, J. F., VERHAAR, J. A., DE CLERCK, L. S. & SOMVILLE, J. 2010. The infrapatellar fat pad should be considered as an active osteoarthritic joint tissue: a narrative review. *Osteoarthritis Cartilage,* 18**,** 876-82.

COUSSENS, L. M. & WERB, Z. 2002. Inflammation and cancer. *Nature,* 420**,** 860-7.
CREAMER, P. & HOCHBERG, M. C. 1997. Osteoarthritis. *Lancet,* 350**,** 503-8.

DAS GRACAS COELHO DE SOUZA, M., KRAEMER-AGUIAR, L. G. & BOUSKELA, E. 2016. Inflammation-induced microvascular dysfunction in obesity - A translational approach. *Clin Hemorheol Microcirc,* 64**,** 645-654.

DAVIES-TUCK, M. L., WLUKA, A. E., FORBES, A., WANG, Y., ENGLISH, D. R., GILES, G. G. & CICUTTINI, F. 2009. Smoking is associated with increased cartilage loss and persistence of bone marrow lesions over 2 years in community-based individuals. *Rheumatology (Oxford),* 48**,** 1227-31.

DAVIES, D. V. 1950. The structure and functions of the synovial membrane. *Br Med J,* 1**,** 92-5.

DE CIUCEIS, C., PORTERI, E., RIZZONI, D., CORBELLINI, C., LA BORIA, E., BOARI, G. E., PILU, A., MITTEMPERGHER, F., DI BETTA, E., CASELLA, C., NASCIMBENI, R., ROSEI, C. A., RUGGERI, G., CAIMI, L. & ROSEI, E. A. 2011. Effects of weight loss on structural and functional alterations of subcutaneous small arteries in obese patients. *Hypertension,* 58**,** 29-36.

DEANFIELD, J. E., HALCOX, J. P. & RABELINK, T. J. 2007. Endothelial function and dysfunction: testing and clinical relevance. *Circulation,* 115**,** 1285-95.

DEN HAAN, J. M., ARENS, R. & VAN ZELM, M. C. 2014. The activation of the adaptive immune system: cross-talk between antigen-presenting cells, T cells and B cells. *Immunol Lett,* 162**,** 103-12.

DEN UIL, C. A., KLIJN, E., LAGRAND, W. K., BRUGTS, J. J., INCE, C., SPRONK, P. E. & SIMOONS, M. L. 2008. The microcirculation in health and critical disease. *Prog Cardiovasc Dis,* 51**,** 161-70.

DING, C., CICUTTINI, F., BLIZZARD, L. & JONES, G. 2007. Smoking interacts with family history with regard to change in knee cartilage volume and cartilage defect development. *Arthritis Rheum,* 56**,** 1521-8.

DING, L., HEYING, E., NICHOLSON, N., STROUD, N. J., HOMANDBERG, G. A., BUCKWALTER, J. A., GUO, D. & MARTIN, J. A. 2010. Mechanical impact induces cartilage degradation via mitogen activated protein kinases. *Osteoarthritis Cartilage,* 18**,** 1509-17.

DUBE, C. E., LIU, S. H., DRIBAN, J. B., MCALINDON, T. E., EATON, C. B. & LAPANE, K. L. 2016. The relationship between smoking and knee osteoarthritis in the Osteoarthritis Initiative. *Osteoarthritis Cartilage,* 24**,** 465-72.

DVORAK, H. F. 2005. Angiogenesis: update 2005. *J Thromb Haemost,* 3**,** 1835-42.
EDWARDS, J., LEIGH, R. & CAMBRIDGE, G. 1997. Expression of molecules involved in B lymphocyte survival and differentiation by synovial fibroblasts. *Clinical & Experimental Immunology,* 108**,** 407-414.

FANG, L., GUO, F., ZHOU, L., STAHL, R. & GRAMS, J. 2015. The cell size and distribution of adipocytes from subcutaneous and visceral fat is associated with type 2 diabetes mellitus in humans. *Adipocyte,* 4**,** 273-9.

FARAHAT, M. N., YANNI, G., POSTON, R. & PANAYI, G. S. 1993. Cytokine expression in synovial membranes of patients with rheumatoid arthritis and osteoarthritis. *Ann Rheum Dis,* 52**,** 870-5.

FEJER, R. & RUHE, A. 2012. What is the prevalence of musculoskeletal problems in the elderly population in developed countries? A systematic critical literature review. *Chiropr Man Therap,* 20**,** 31.

FELETOU, M., TANG, E. H. & VANHOUTTE, P. M. 2008. Nitric oxide the gatekeeper of endothelial vasomotor control. *Front Biosci,* 13**,** 4198-217.

FELSON, D. T. & ZHANG, Y. 1998. An update on the epidemiology of knee and hip osteoarthritis with a view to prevention. *Arthritis Rheum,* 41**,** 1343-55.

FERNANDES-SANTOS, C., CARNEIRO, R. E., DE SOUZA MENDONCA, L., AGUILA, M. B. & MANDARIM-DE-LACERDA, C. A. 2009. Pan-PPAR agonist beneficial effects in overweight mice fed a high-fat high-sucrose diet. *Nutrition,* 25**,** 818-27.

FINA, L., MOLGAARD, H. V., ROBERTSON, D., BRADLEY, N. J., MONAGHAN, P., DELIA, D., SUTHERLAND, D. R., BAKER, M. A. & GREAVES, M. F. 1990. Expression of the CD34 gene in vascular endothelial cells. *Blood,* 75**,** 2417-26.

FRASER, I. P., KOZIEL, H. & EZEKOWITZ, R. A. 1998. The serum mannose-binding protein and the macrophage mannose receptor are pattern recognition molecules that link innate and adaptive immunity. *Semin Immunol,* 10**,** 363-72.

FUCHS, E. & WEBER, K. 1994. Intermediate filaments: structure, dynamics, function, and disease. *Annu Rev Biochem,* 63**,** 345-82.

GAO, Y. J. 2007. Dual modulation of vascular function by perivascular adipose tissue and its potential correlation with adiposity/lipoatrophy-related vascular dysfunction. *Curr Pharm Des,* 13**,** 2185-92.

GOLDBLATT, J. P. & RICHMOND, J. C. 2003. Anatomy and biomechanics of the knee. *Operative Techniques in Sports Medicine,* 11**,** 172-186.

GOLDRING, M. B. & OTERO, M. 2011. Inflammation in osteoarthritis. *Curr Opin Rheumatol,* 23**,** 471-8.

GOLDRING, S. R. & GOLDRING, M. B. 2006. Clinical aspects, pathology and pathophysiology of osteoarthritis. *J Musculoskelet Neuronal Interact,* 6**,** 376-8.
GRANGER, D. N. & SENCHENKOVA, E. 2010. Integrated Systems Physiology—From Cell to Function. *Inflammation and the Microcirculation.* San Rafael (CA): Morgan & Claypool Life Sciences Copyright (c) 2010 by Morgan & Claypool Life Sciences.

HAN, Q.-F., WU, L., LI, T., MENG, X.-Y. & YAO, H.-C. 2016. There is a link between carotid intima media thickness and coronary artery disease: It might be inflammation. *International Journal of Cardiology,* 203, 1144-1145.

HANNAH, M. J., WILLIAMS, R., KAUR, J., HEWLETT, L. J. & CUTLER, D. F. 2002. Biogenesis of Weibel-Palade bodies. *Semin Cell Dev Biol,* 13**,** 313-24.

HARAOUI, B., PELLETIER, J. P., CLOUTIER, J. M., FAURE, M. P. & MARTEL-PELLETIER, J. 1991. Synovial membrane histology and immunopathology in rheumatoid arthritis and osteoarthritis. In vivo effects of antirheumatic drugs. *Arthritis Rheum,* 34**,** 153-63.

HEALY, L., MAY, G., GALE, K., GROSVELD, F., GREAVES, M. & ENVER, T. 1995. The stem cell antigen CD34 functions as a regulator of hemopoietic cell adhesion. *Proc Natl Acad Sci U S A,* 92**,** 12240-4.

HEIDARI, B. 2011. Knee osteoarthritis prevalence, risk factors, pathogenesis and features: Part I. *Caspian J Intern Med,* 2**,** 205-12.

HERRMANN, H., BAR, H., KREPLAK, L., STRELKOV, S. V. & AEBI, U. 2007. Intermediate filaments: from cell architecture to nanomechanics. *Nat Rev Mol Cell Biol,* 8**,** 562-73. HGNC:1693.

HICKLIN, D. J. & ELLIS, L. M. 2005. Role of the vascular endothelial growth factor pathway in tumor growth and angiogenesis. *J Clin Oncol,* 23**,** 1011-27.

HOEFSMIT, E. C., DUIJVESTIJN, A. M. & KAMPERDIJK, E. W. 1982. Relation between langerhans cells, veiled cells, and interdigitating cells. *Immunobiology,* 161**,** 255-65.

HUANG, M. F., LIN, W. L. & MA, Y. C. 2005. A study of reactive oxygen species in mainstream of cigarette. *Indoor Air,* 15**,** 135-40.

HUI, A. Y., MCCARTY, W. J., MASUDA, K., FIRESTEIN, G. S. & SAH, R. L. 2012. A systems biology approach to synovial joint lubrication in health, injury, and disease. *Wiley Interdiscip Rev Syst Biol Med,* 4**,** 15-37.

IVASKA, J., PALLARI, H. M., NEVO, J. & ERIKSSON, J. E. 2007. Novel functions of vimentin in cell adhesion, migration, and signaling. *Exp Cell Res,* 313**,** 2050-62.

JAFFE, E. A., NACHMAN, R. L., BECKER, C. G. & MINICK, C. R. 1973. Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. *J Clin Invest,* 52**,** 2745-56.

JAY, G. D., BRITT, D. E. & CHA, C. J. 2000. Lubricin is a product of megakaryocyte stimulating factor gene expression by human synovial fibroblasts. *J Rheumatol,* 27**,** 594-600.

JOSÉ A. RAMOS-VARA, M. K., , TIMOTHY BASZLER, LAURA BLIVEN, BRUCE BRODERSEN, BRIAN CHELACK, KEITH WEST, STEFANIE CZUB, FABIO DEL PIERO, SHARON DIAL, E. J. EHRHART, TANYA GRAHAM, LISA MANNING, DANIEL PAULSEN, VICTOR E. VALLI 2008. Suggested Guidelines for Immunohistochemical Techniques in Veterinary Diagnostic Laboratories. *Journal of Veterinary Diagnostic Investigation,* 20**,** 393 - 413

KANNEGANTI, T.-D. & DIXIT, V. D. 2012. Immunological complications of obesity. *Nat Immunol,* 13**,** 707-712.

KAPOOR, M., MARTEL-PELLETIER, J., LAJEUNESSE, D., PELLETIER, J. P. & FAHMI, H. 2011. Role of proinflammatory cytokines in the pathophysiology of osteoarthritis. *Nat Rev Rheumatol,* 7**,** 33-42.

KARIN, M. 2006. Nuclear factor-kappaB in cancer development and progression. *Nature,* 441**,** 431-6.

KIM, H. A., CHO, M. L., CHOI, H. Y., YOON, C. S., JHUN, J. Y., OH, H. J. & KIM, H. Y. 2006. The catabolic pathway mediated by Toll-like receptors in human osteoarthritic chondrocytes. *Arthritis Rheum,* 54**,** 2152-63.

KRENN, V., MORAWIETZ, L., BURMESTER, G. R., KINNE, R. W., MUELLER-LADNER, U., MULLER, B. & HAUPL, T. 2006. Synovitis score: discrimination between chronic low-grade and high-grade synovitis. *Histopathology,* 49**,** 358-64.

KUNISCH, E., FUHRMANN, R., ROTH, A., WINTER, R., LUNGERSHAUSEN, W. & KINNE, R. W. 2004. Macrophage specificity of three anti-CD68 monoclonal antibodies (KP1, EBM11, and PGM1) widely used for immunohistochemistry and flow cytometry. *Ann Rheum Dis,* 63**,** 774-84.

LAWRENCE, R. C., FELSON, D. T., HELMICK, C. G., ARNOLD, L. M., CHOI, H., DEYO, R. A., GABRIEL, S., HIRSCH, R., HOCHBERG, M. C., HUNDER, G. G., JORDAN, J. M., KATZ, J. N., KREMERS, H. M. & WOLFE, F. 2008. Estimates of the prevalence of arthritis and other rheumatic conditions in the United States. Part II. *Arthritis Rheum,* 58**,** 26-35.

LEBIEN, T. W. & TEDDER, T. F. 2008. B lymphocytes: how they develop and function. *Blood,* 112**,** 1570-80.

LEE, J., TANEJA, V. & VASSALLO, R. 2012. Cigarette smoking and inflammation: cellular and molecular mechanisms. *J Dent Res,* 91**,** 142-9.

LEE, Y. C. 2005. The involvement of VEGF in endothelial permeability: a target for anti-inflammatory therapy. *Curr Opin Investig Drugs,* 6**,** 1124-30.

LEVICK, J. R. 1995. Microvascular architecture and exchange in synovial joints. *Microcirculation,* 2**,** 217-33.

LIBBY, P., OKAMOTO, Y., ROCHA, V. Z. & FOLCO, E. 2010. Inflammation in atherosclerosis: transition from theory to practice. *Circ J,* 74**,** 213-20.

LINGEN, M. W. 2001. Role of leukocytes and endothelial cells in the development of angiogenesis in inflammation and wound healing. *Arch Pathol Lab Med,* 125**,** 67-71.

LIU, Y. C., ZOU, X. B., CHAI, Y. F. & YAO, Y. M. 2014. Macrophage polarization in inflammatory diseases. *Int J Biol Sci,* 10**,** 520-9.

LOESER, R. F., GOLDRING, S. R., SCANZELLO, C. R. & GOLDRING, M. B. 2012. Osteoarthritis: a disease of the joint as an organ. *Arthritis Rheum,* 64**,** 1697-707.
LORENZ, H. & RICHTER, W. 2006. Osteoarthritis: cellular and molecular changes in degenerating cartilage. *Prog Histochem Cytochem,* 40**,** 135-63.

MAPP, P. I. & WALSH, D. A. 2012. Mechanisms and targets of angiogenesis and nerve growth in osteoarthritis. *Nat Rev Rheumatol,* 8**,** 390-8.

MARTEL-PELLETIER, J. 2004. Pathophysiology of osteoarthritis. *OsteoArthritis and Cartilage* 12.

MARTINEK, V. 2003. Anatomy and pathophysiology of articular cartilage. *Dtsch Z Sportmed* 166–70.

MEDZHITOV, R. 2007. Recognition of microorganisms and activation of the immune response. *Nature,* 449**,** 819-26.

MEDZHITOV, R. & JANEWAY, C. 2000. Innate immune recognition: mechanisms and pathways. *Immunological reviews,* 173**,** 89-97.

MELDRUM, D. R., MORRIS, M. A. & GAMBONE, J. C. 2017. Obesity pandemic: causes, consequences, and solutions-but do we have the will? *Fertil Steril,* 107**,** 833-839.

MESSIER, S. P., GUTEKUNST, D. J., DAVIS, C. & DEVITA, P. 2005. Weight loss reduces knee-joint loads in overweight and obese older adults with knee osteoarthritis. *Arthritis Rheum,* 52**,** 2026-32.

MESSIER, S. P., LOESER, R. F., MITCHELL, M. N., VALLE, G., MORGAN, T. P., REJESKI, W. J. & ETTINGER, W. H. 2000. Exercise and weight loss in obese older adults with knee osteoarthritis: a preliminary study. *J Am Geriatr Soc,* 48**,** 1062-72.

MIAO, C. Y. & LI, Z. Y. 2012. The role of perivascular adipose tissue in vascular smooth muscle cell growth. *Br J Pharmacol,* 165**,** 643-58.

MICHAEL, J. W., SCHLUTER-BRUST, K. U. & EYSEL, P. 2010. The epidemiology, etiology, diagnosis, and treatment of osteoarthritis of the knee. *Dtsch Arztebl Int,* 107**,** 152-62.

MIETTINEN, M., LINDENMAYER, A. E. & CHAUBAL, A. 1994. Endothelial cell markers CD31, CD34, and BNH9 antibody to H- and Y-antigens--evaluation of their specificity and sensitivity in the diagnosis of vascular tumors and comparison with von Willebrand factor. *Mod Pathol,* 7**,** 82-90.

MOSIER, D. E. & COPPLESON, L. W. 1968. A THREE-CELL INTERACTION REQUIRED FOR THE INDUCTION OF THE PRIMARY IMMUNE RESPONSE in vitro. *Proc Natl Acad Sci U S A,* 61**,** 542-7.

MUCKE, J., HOYER, A., BRINKS, R., BLECK, E., PAULY, T., SCHNEIDER, M. & VORDENBAUMEN, S. 2016. Inhomogeneity of immune cell composition in the synovial sublining: linear mixed modelling indicates differences in distribution and spatial decline of CD68+ macrophages in osteoarthritis and rheumatoid arthritis. *Arthritis Res Ther,* 18**,** 170.

MULLER, W. A. 2003. Leukocyte-endothelial-cell interactions in leukocyte transmigration and the inflammatory response. *Trends Immunol,* 24**,** 327-34.

MURDOCH, C., MUTHANA, M. & LEWIS, C. E. 2005. Hypoxia regulates macrophage functions in inflammation. *J Immunol,* 175**,** 6257-63.

MYERS, S. L., BRANDT, K. D., EHLICH, J. W., BRAUNSTEIN, E. M., SHELBOURNE, K. D., HECK, D. A. & KALASINSKI, L. A. 1990. Synovial inflammation in patients with early osteoarthritis of the knee. *J Rheumatol,* 17**,** 1662-9.

DR. ASHISH N DEBROY 2012. Available: http://rsbweb.nih.gov/ij/docs/examples/stained-sections/index.html [Accessed 10-06-2013].

NAIK, S. H., PERIE, L., SWART, E., GERLACH, C., VAN ROOIJ, N., DE BOER, R. J. & SCHUMACHER, T. N. 2013. Diverse and heritable lineage imprinting of early haematopoietic progenitors. *Nature,* 496**,** 229-32.

NAIR, A., KANDA, V., BUSH-JOSEPH, C., VERMA, N., CHUBINSKAYA, S., MIKECZ, K., GLANT, T. T., MALFAIT, A. M., CROW, M. K., SPEAR, G. T., FINNEGAN, A. & SCANZELLO, C. R. 2012. Synovial fluid from patients with early osteoarthritis modulates fibroblast-like synoviocyte responses to toll-like receptor 4 and toll-like receptor 2 ligands via soluble CD14. *Arthritis Rheum,* 64**,** 2268-77.

NALDINI, A. & CARRARO, F. 2005. Role of inflammatory mediators in angiogenesis. *Curr Drug Targets Inflamm Allergy,* 4**,** 3-8.

NATHAN, C. 2002. Points of control in inflammation. *Nature,* 420**,** 846-52.

NATIONAL CLINICAL GUIDELINE, C. 2014. National Institute for Health and Clinical Excellence: Guidance. *Osteoarthritis: Care and Management in Adults.* London: National Institute for Health and Care Excellence (UK) Copyright (c) National Clinical Guideline Centre, 2014.

NEEFJES, J., JONGSMA, M. L., PAUL, P. & BAKKE, O. 2011. Towards a systems understanding of MHC class I and MHC class II antigen presentation. *Nat Rev Immunol,* 11**,** 823-36.

NIELSEN, J. S. & MCNAGNY, K. M. 2009. CD34 is a key regulator of hematopoietic stem cell trafficking to bone marrow and mast cell progenitor trafficking in the periphery. *Microcirculation,* 16**,** 487-96.

OUCHI, N., PARKER, J. L., LUGUS, J. J. & WALSH, K. 2011. Adipokines in inflammation and metabolic disease. *Nat Rev Immunol,* 11**,** 85-97.

PARASHAR;, P. & HELLIWELL, M. R. Y. N. S. F. T. 2012 *Inflammation and Vascular Recruitment in Synovitis of the Shoulder* [Online]. [Accessed S1226].

PEARLE, A. D., SCANZELLO, C. R., GEORGE, S., MANDL, L. A., DICARLO, E. F., PETERSON, M., SCULCO, T. P. & CROW, M. K. 2007. Elevated high-sensitivity C-reactive protein levels are associated with local inflammatory findings in patients with osteoarthritis. *Osteoarthritis Cartilage,* 15**,** 516-23.

PESSLER, F., CHEN, L. X., DAI, L., GOMEZ-VAQUERO, C., DIAZ-TORNE, C., PAESSLER, M. E., SCANZELLO, C., CAKIR, N., EINHORN, E. & SCHUMACHER, H. R. 2008a. A histomorphometric analysis of synovial biopsies from individuals with Gulf War Veterans' Illness and joint pain compared to normal and osteoarthritis synovium. *Clin Rheumatol,* 27**,** 1127-34.

PESSLER, F., DAI, L., DIAZ-TORNE, C., GOMEZ-VAQUERO, C., PAESSLER, M. E., ZHENG, D. H., EINHORN, E., RANGE, U., SCANZELLO, C. & SCHUMACHER, H. R. 2008b. The synovitis of "non-inflammatory" orthopaedic arthropathies: a quantitative histological and immunohistochemical analysis. *Ann Rheum Dis,* 67**,** 1184-7.

PHILIP, M., ROWLEY, D. A. & SCHREIBER, H. 2004. Inflammation as a tumor promoter in cancer induction. *Semin Cancer Biol,* 14**,** 433-9.

POZGAN, U., CAGLIC, D., ROZMAN, B., NAGASE, H., TURK, V. & TURK, B. 2010. Expression and activity profiling of selected cysteine cathepsins and matrix metalloproteinases in synovial fluids from patients with rheumatoid arthritis and osteoarthritis. *Biol Chem,* 391**,** 571-9.

PUENPATOM, R. A. & VICTOR, T. W. 2009. Increased prevalence of metabolic syndrome in individuals with osteoarthritis: an analysis of NHANES III data. *Postgrad Med,* 121**,** 9-20.

RAMACHANDRAN, M. 2007. *BASIC ORTHOPAEDIC SCIENCES The Stanmore Guide*, Hodder Arnold.

REVELL, P., AL-SAFFAR, N., FISH, S. & OSEI, D. 1995. Extracellular matrix of the synovial intimal cell layer. *Annals of the rheumatic diseases,* 54**,** 404.

RILEY, J. K. & SLIWKOWSKI, M. X. 2000. CD20: a gene in search of a function. *Semin Oncol,* 27**,** 17-24.

ROCHA, V. Z. & LIBBY, P. 2009. Obesity, inflammation, and atherosclerosis. *Nat Rev Cardiol,* 6**,** 399-409.

ROSENTHAL, A. K. 2011. Crystals, inflammation, and osteoarthritis. *Curr Opin Rheumatol,* 23**,** 170-3.

SADLER, J. E. 1998. Biochemistry and genetics of von Willebrand factor. *Annu Rev Biochem,* 67**,** 395-424.

SANDELL, L. J. & AIGNER, T. 2001. Articular cartilage and changes in arthritis. An introduction: cell biology of osteoarthritis. *Arthritis Res,* 3**,** 107-13.

SCHOFIELD, D. J., SHRESTHA, R. N., PERCIVAL, R., PASSEY, M. E., CALLANDER, E. J. & KELLY, S. J. 2013. The personal and national costs of lost labour force participation due to arthritis: an economic study. *BMC Public Health,* 13**,** 188.

SCHRAML, B. U., VAN BLIJSWIJK, J., ZELENAY, S., WHITNEY, P. G., FILBY, A., ACTON, S. E., ROGERS, N. C., MONCAUT, N., CARVAJAL, J. J. & E SOUSA, C. R. 2013. Genetic tracing via DNGR-1 expression history defines dendritic cells as a hematopoietic lineage. *Cell,* 154**,** 843-858.

SCHUMACHER, B. L., BLOCK, J. A., SCHMID, T. M., AYDELOTTE, M. B. & KUETTNER, K. E. 1994. A novel proteoglycan synthesized and secreted by chondrocytes of the superficial zone of articular cartilage. *Arch Biochem Biophys,* 311**,** 144-52.

SCHURZ, J. & RIBITSCH, V. 1987. Rheology of synovial fluid. *Biorheology,* 24**,** 385-99.

SELLAM, J. & BERENBAUM, F. 2010. The role of synovitis in pathophysiology and clinical symptoms of osteoarthritis. *Nat Rev Rheumatol,* 6**,** 625-35.

SIDNEY, L. E., BRANCH, M. J., DUNPHY, S. E., DUA, H. S. & HOPKINSON, A. 2014. Concise review: evidence for CD34 as a common marker for diverse progenitors. *Stem Cells,* 32**,** 1380-9.

SMITH, M. D. 2011. The normal synovium. *Open Rheumatol J,* 5**,** 100-6.
SMITH, M. D., BARG, E., WEEDON, H., PAPENGELIS, V., SMEETS, T., TAK, P. P., KRAAN, M., COLEMAN, M. & AHERN, M. J. 2003. Microarchitecture and protective mechanisms in synovial tissue from clinically and arthroscopically normal knee joints. *Annals of the Rheumatic Diseases,* 62**,** 303-307.

SMITH, M. D., TRIANTAFILLOU, S., PARKER, A., YOUSSEF, P. P. & COLEMAN, M. 1997. Synovial membrane inflammation and cytokine production in patients with early osteoarthritis. *J Rheumatol,* 24**,** 365-71.

SPECTOR, T. D., HART, D. J., NANDRA, D., DOYLE, D. V., MACKILLOP, N., GALLIMORE, J. R. & PEPYS, M. B. 1997. Low-level increases in serum C-reactive protein are present in early osteoarthritis of the knee and predict progressive disease. *Arthritis Rheum,* 40**,** 723-7.

STAHL, P. D. & EZEKOWITZ, R. A. 1998. The mannose receptor is a pattern recognition receptor involved in host defense. *Curr Opin Immunol,* 10**,** 50-5.
STASHENKO, P., NADLER, L. M., HARDY, R. & SCHLOSSMAN, S. F. 1980. Characterization of a human B lymphocyte-specific antigen. *J Immunol,* 125**,** 1678-85.

STEIN, J. H., KORCARZ, C. E., HURST, R. T., LONN, E., KENDALL, C. B., MOHLER, E. R., NAJJAR, S. S., REMBOLD, C. M. & POST, W. S. 2008. Use of carotid ultrasound to identify subclinical vascular disease and evaluate cardiovascular disease risk: a consensus statement from the American Society of Echocardiography Carotid Intima-Media Thickness Task Force. Endorsed by the Society for Vascular Medicine. *J Am Soc Echocardiogr,* 21**,** 93-111; quiz 189-90.

SUN, A. R., FRIIS, T., SEKAR, S., CRAWFORD, R., XIAO, Y. & PRASADAM, I. 2016. Is Synovial Macrophage Activation the Inflammatory Link Between Obesity and Osteoarthritis? *Curr Rheumatol Rep,* 18**,** 57.

TAYLOR, C. R. & BURNS, J. 1974. The demonstration of plasma cells and other immunoglobulin-containing cells in formalin-fixed, paraffin-embedded tissues using peroxidase-labelled antibody. *J Clin Pathol,* 27**,** 14-20.

TEDDER, T. F., STREULI, M., SCHLOSSMAN, S. F. & SAITO, H. 1988. Isolation and structure of a cDNA encoding the B1 (CD20) cell-surface antigen of human B lymphocytes. *Proc Natl Acad Sci U S A,* 85**,** 208-12.

TINDLE, R. W., NICHOLS, R. A., CHAN, L., CAMPANA, D., CATOVSKY, D. & BIRNIE, G. D. 1985. A novel monoclonal antibody BI-3C5 recognises myeloblasts and non-B non-T lymphoblasts in acute leukaemias and CGL blast crises, and reacts with immature cells in normal bone marrow. *Leuk Res,* 9**,** 1-9.

TISELIUS, A. & KABAT, E. A. 1938. ELECTROPHORESIS OF IMMUNE SERUM. *Science,* 87**,** 416-7.

TORZILLI, P. A., BHARGAVA, M., PARK, S. & CHEN, C. T. 2010. Mechanical load inhibits IL-1 induced matrix degradation in articular cartilage. *Osteoarthritis Cartilage,* 18**,** 97-105.

UK, A. R. 2017. *The State of Musculoskeletal Health 2017* [Online]. Arthritis Research UK [Accessed].

VAN DER KRAAN, P. M. 2013a. Relevance of zebrafish as an OA research model. *Osteoarthritis Cartilage,* 21**,** 261-2.

VAN DER KRAAN, P. M. 2013b. Understanding developmental mechanisms in the context of osteoarthritis. *Curr Rheumatol Rep,* 15**,** 333.

VAN LENT, P. L., BLOM, A. B., SCHELBERGEN, R. F., SLOETJES, A., LAFEBER, F. P., LEMS, W. F., CATS, H., VOGL, T., ROTH, J. & VAN DEN BERG, W. B. 2012. Active involvement of alarmins S100A8 and S100A9 in the regulation of synovial activation and joint destruction during mouse and human osteoarthritis. *Arthritis Rheum,* 64**,** 1466-76.

VERGUNST, C. E., VAN DE SANDE, M. G., LEBRE, M. C. & TAK, P. P. 2005. The role of chemokines in rheumatoid arthritis and osteoarthritis. *Scand J Rheumatol,* 34**,** 415-25.

VINAY, K., ABDUL K, A. & JON, A. 2015. *Robbins and Cotran pathologic basis of disease,* Philadelphia, Elsevier/Saunders.

VINAY KUMAR, A. K. A., JON ASTER 9th Edition. Robbins Basic Pathology.
VINCENT, H. K., HEYWOOD, K., CONNELLY, J. & HURLEY, R. W. 2012. Obesity and weight loss in the treatment and prevention of osteoarthritis. *Pm r,* 4**,** S59-67.

VYAS, J. M., VAN DER VEEN, A. G. & PLOEGH, H. L. 2008. The known unknowns of antigen processing and presentation. *Nat Rev Immunol,* 8**,** 607-18.

WANG, Q., ROZELLE, A. L., LEPUS, C. M., SCANZELLO, C. R., SONG, J. J., LARSEN, D. M., CRISH, J. F., BEBEK, G., RITTER, S. Y., LINDSTROM, T. M., HWANG, I., WONG, H. H., PUNZI, L., ENCARNACION, A., SHAMLOO, M., GOODMAN, S. B., WYSS-CORAY, T., GOLDRING, S. R., BANDA, N. K., THURMAN, J. M., GOBEZIE, R., CROW, M. K., HOLERS, V. M., LEE, D. M. & ROBINSON, W. H. 2011. Identification of a central role for complement in osteoarthritis. *Nat Med,* 17**,** 1674-9.

WEIBEL, E. R. & PALADE, G. E. 1964. NEW CYTOPLASMIC COMPONENTS IN ARTERIAL ENDOTHELIA. *J Cell Biol,* 23**,** 101-12.

WILKINSON, L. S. & EDWARDS, J. C. 1989. Microvascular distribution in normal human synovium. *J Anat,* 167**,** 129-36.

WILKINSON, L. S., PITSILLIDES, A. A., WORRALL, J. G. & EDWARDS, J. C. 1992. Light microscopic characterization of the fibroblast-like synovial intimal cell (synoviocyte). *Arthritis Rheum,* 35**,** 1179-84.

WOLLENBERG, A. & BIEBER, T. 2002. Antigen presenting cells. *Atopic dermatitis. Marcel Dekker, New York***,** 267-283.

WOLLHEIM, F. A. 1999. Serum markers of articular cartilage damage and repair. *Rheum Dis Clin North Am,* 25**,** 417-32, viii.

XU, H., EDWARDS, J., BANERJI, S., PREVO, R., JACKSON, D. G. & ATHANASOU, N. A. 2003. Distribution of lymphatic vessels in normal and arthritic human synovial tissues. *Ann Rheum Dis,* 62**,** 1227-9.

ZHUO, Q., YANG, W., CHEN, J. & WANG, Y. 2012. Metabolic syndrome meets osteoarthritis. *Nat Rev Rheumatol,* 8**,** 729-37.