5.1 DISCUSSION

Osteoarthritis (OA) is the leading cause of chronic disability, common sites being the knee and hip (Grazio and Balen, 2009). The strong association between age and increasing incidence of osteoarthritis (OA) marks OA as an age related disease (James and Joseph, 2002). In the present study, out of 75 OA patients, the incidence of patients suffering from knee OA were found to be highest between the age group 65-69 years (32%) followed by the age group between 60-64 years (24%). Several studies has observed that the incidence of knee OA increases with age and further increases with longer lifetime and higher average weight of the population (Bliddal and Christensen, 2009; Zhang and Jordan, 2010). Age dependent changes in articular cartilage, increases the risk of the synovial joint degeneration causing the clinical syndrome of OA and in addition, these changes may adversely affect the outcomes of attempts to repair or regenerate articular cartilage (James and Joseph, 2002). The age group between 70-74 years were less (7%) in comparison with other age groups and it could be due to the fact that the patients between this age group may be ignorant regarding their health.

Race and ethnicity, along with gender, are important factors in determining both short-term and lifetime risk of developing symptomatic knee osteoarthritis (ACR, 2012). In the study, more females (70%) suffered from knee OA as compared to males (30%). Population based studies in developed and developing countries have consistently reported a higher prevalence of radiographic knee osteoarthritis (knee ROA) in women than in men (WHO, 2003; Andersen et al., 1999; Thomas et al., 2004; Dillon et al., 2006). Loss of estrogen at the time of menopause increases the women’s risk of getting OA (Spector and Campion, 1989) and also influences the activity of the
joint tissues by acting at multiple levels through complex molecular pathways (Roman et al. 2009).

From the ethnic groups, highest number of patients with knee OA were observed in the Bhutias (42.6%) followed by the Lepchas (29.3%) whereas the Nepalis being the major ethnic group were found to be only 20% and rest (8%) belonged from other non ethnic communities of Sikkim. Nutritional and genetic factor may be one of the causes. Calcium is an essential bone forming mineral needed for normal skeletal development (Davies et al. 2005). One of the study have demonstrated that there was threefold increase risk of progression of OA for people in the lower decile of vitamin C and D blood levels (Wolf and Pfleger, 2003). Results from the Framingham Study indicate that high intake of vitamin C may be associated with a lower risk of knee osteoarthritis progression, but does not appear to prevent the onset of disease (McAlindon et al. 1996). Vitamin D obtained from dietary sources or sun exposure is required for normal bone metabolism; low levels may affect bone repair and muscle strength, predisposing to progression of osteoarthritis (Felson et al. 2000). There is evidence from longitudinal studies that low dietary and serum levels of vitamin D may be associated with the development and progression of knee osteoarthritis (McAlindon et al. 1996; McAlindon and Felson, 1997).

The presence of endogenous OP-1 in the human synovial fluid have been studied (Chubinskaya et al. 2006). OP-1 has been implicated to have a wide range of anabolic and anti-catabolic activities (Rueger et al. 2004). In the present study, the sample having highest OP-1 concentration (No. 25) among clinically diagnosed 75 osteoarthritic patients was isolated and experimental mice was immunized
intraperitoneally to produce polyclonal antibody (anti-OP-1). The antigenic fraction OP-1(f) isolated by SDS-PAGE showed a molecular weight of around 36kD among 12 SDS-PAGE fractions. Few studies showed that the molecular weight of OP-1 in osteoarthritic patients to be around 18kD and 36kD (Collins, 1949; Chubinskaya et al. 2002). The antibody, anti-OP-1(f) IgG was found to be useful in detecting the OP-1 in the synovial fluid of osteoarthritic patients. By preparing the standards of known concentration of OP-1 (78.4 ug/ml) we have also attempted to find the concentration of OP-1 in the synovial fluid of 75 osteoarthritic patients. It was observed that the level of the OP-1 appeared to be high when compared with normal values of about 50ng/ml (Merrihew et al. 2003; Chubinskaya et al., 2002). Few studies have also shown an increase in the level of OP-1 in osteoarthritic patients (Chubinskaya et al. 2002; Honsawek et al. 2009). Honsawek and his colleagues (2009) demonstrated the presence of OP-1 in plasma and synovial fluid of OA patients and correlated among the two body fluids and suggested that increase level of OP-1 is associated with the progressive damage of the joint in knee OA. Similarly, few of the studies have reported an increase levels of OP-1 in synovial fluid and tissues after trauma (Hurtig; Hurtig and Chubinskaya, 2004), after an arthroscopy incision (Fahlgren et al. 2006) or induction of OA (Muehleman et al. 2002) indicating as a part of normal reparative response. Kaps et al (2002) demonstrated that the chondrocytes which expresses BMP-7 have shown to suppress the growth of destructive fibrous connective tissue (pannus) so that BMP-7 may be useful in inflammatory arthritis which may be considered as a reparative process. In addition, BMP-7 might be derived from any of the other connective tissues of the synovial joint like the meniscus, ligament, and tendon—since it has been identified in all connective tissues of the joint (Rueger et al. 2004). The elevation of OP-1 in both the body fluids may suggest an enhanced
local and systemic production of OP-1 levels in primary knee OA. Firstly, high levels in synovial fluid may be either due to the BMP-7 residing in the extracellular matrix or by increased production or both. Secondly, BMP-7 could be released by the synovial cell and chondrocytes in the local tissues such as those of synovial membrane and articular cartilage in an autocrine or paracrine manner leading to the increase in the level of BMP-7 in the synovial fluid. Similarly, one of the study also reported that OP-1 level increases in response to inflammation (Chubinskaya et al. 2006) in OA.

Significant positive correlation was found between OP-1 level in synovial fluid and the severity of OA and the result was found to be similar as observed by Honsawek et al. (2009). We also observed that the synovial fluid level of OP-1 in KL grade 3 & 4 was found to be higher than those of grade 1 and 2 but the difference was statistically insignificant.

Inflammation and oxidative stress are believed to function as degenerative mechanism in the development and progression of OA (Rao et al. 2005). Different studies worldwide have shown an alteration in redox status and the shift towards oxidative stress leading to decrease in antioxidant levels as they are oxidized by the free radical and this is the protective response of the body to any oxidative damage. Synovial cavity damage has been associated with oxidative stress by some studies (Hooiveld et al. 2001). Chen and his colleagues (1989) reported a high predisposition of free radical release and tissue damage in OA. In the present study, significant decrease in antioxidant enzymes SOD and GPx is in consort with few other studies (Maneesh et al. 2005; Kalaci et al. 2007; Regan et al. 2005; Gavriilidis et al. 2013) indicating increase in the oxidative stress, ultimately leading to oxidative damage. Similarly, one of the
study has demonstrated a decreased in expression of all three SOD isoforms (copper/zinc (Cu/Zn)-SOD, manganese (Mn)-SOD, and extracellular (EC)-SOD), at the transcriptional level (Scott et al. 2010). Whereas few studies have also reported an increase in SOD level in the synovial fluid of OA patients and correlated positively with the KL grading (Dawn et al. 2013). Similar results of raised SOD activities have been reported in patients with rheumatic diseases (Kaneda, 1982; Maneesh et al. 2005; Ostalowska et al. 2006; Surapaneni et al. 2007). On the other hand, few studies have shown a decrease in synovial fluid level of GPx (Kaneda, 1982; Mezes et al. 1983; Ostalowska et al. 2006; Sutipornpalangkul et al. 2009) indicating that oxidative stress may have a role in pathogenesis of OA. Insignificant activities of GPx was also observed in OA (Karatay et al. 2005). Whereas Surapaneni et al. (2007) reported increase in plasma GPx level. UA being an antioxidant, no significant difference was observed in the present study and this could be due to the complex, selective antioxidant capacity of uric acid (Yuri and Richard, 2008; Banu and Kazim, 2008). Our study findings are consistent with the findings of insignificant changes in UA and no association of serum uric acid in OA (Felson et al. 1988; Bagge et al. 1991; Hart et al. 1995; Sun et al. 2000; Rao et al. 2005). Sun et al. (2000) observed a positive association between serum UA level and generalized OA but not with knee OA.

Hyaluronic acid, being one of the most important biomolecule of articular cartilage is recognized as a marker of synovial inflammation. The concentration of HA was found to be decreased in the study and similar result has been observed (Dahl et al. 1985; Balazs, 1982). It was also demonstrated that the size of HA molecules decreases at the same time as the number of cells in the joint space increases during acute and chronic inflammation of the joint (Dahl et al. 1985; Balazs, 1982, Moreland, 2000; Altman,
The decreased in HA concentration may be due to dilutional effects: dilution of HA in the synovial fluid, reduced hyaluronan synthesis, and free radical degradation (Van den Bekerom et al. 2006). In an earlier study, Sexne et al. (1986) suggested that reduced content of proteoglycan markers in arthritic conditions may be simply due to decreased in cartilage mass. On the other hand increase in serum level of HA has been observed (Turan et al. 2007; M.Sharif et al. 1995). Since HA is widely distributed in the whole body, increase in serum level has also been observed with hepatic (Khan et al. 2007; Hartley et al. 2006), renal (Hallgren et al. 1987) and malignant disease (Wilkinson et al. 2006; Manley and Warren, 1987).

Keratan sulfate has been studied as a promising marker of early cartilage breakdown (Wakiteni et al. 2007; Thonar et al. 1985). The concentration of keratan sulfate was found to be increased in the present study and several studies have projected that KS levels increases in OA (Campion et al. 1991; Mehrabaan et al. 1991). Similar result was observed when KS was measured by 1, 9 dimethyl methylene blue (DMMB) assay in synovial fluid when treated with chondroitin ABC lyase (G.Carroll et al. 1991). Levels of KS in the SF of osteoarthritic patients were found to be much higher than serum and were not found to be correlated (Champion GV et al. 1991). However synovial fluid level of KS was reduced in OA patients when estimated by ELISA, using a monoclonal antibody 5D-4 (C. Belcher et al. 1997; M.Sharif et al. 1996).

Osteoarthritis is the one of most common disease in aging population. It has been earlier demonstrated that the gene and protein expression of OP-1 decreases with aging and with increase in degenerative changes of OA tissue suggesting that OP-1 could be one of the factors responsible for normal homeostasis and matrix integrity.
cartilage (Merrihew *et al.* 2003; Chubinskaya *et al.* 2002; 2007). In the present study, the correlation between OP–1 and age of the osteoarthritic patients was found to be negatively significant. Though OP-1 level increases in OA patients as a part of normal reparative process but as age progresses the level of OP-1 decreases indicating an increase in catabolism of articular cartilage.

Though the level of OP-1 was found to be elevated and UA level was found to be insignificant in osteoarthritic patients but when correlated between them it was found to be positively significant and this may suggest an existing relationship between OP-1 and UA on the development of OA. This may be due to the fact that we had compared the concentration of OP-1 of osteoarthritic patients with the normal value of 50ng/ml (Merrihew *et al.* 2003; Chubinskaya *et al.*, 2002) as we could not compare the OP-1 from the normal synovial fluid due to ethical reason. Hence, in future OP-1 level should be compared from the normal SF fluid to get a true picture of the relationship of OP-1 and UA. Also a sensitivity and specificity analysis would be needed before we can suggest its usefulness in the diagnosis.

We observed an insignificant correlation between OP-1 and cartilage metabolic markers (HA and KS) in OA patients. Significant positive correlation between OP-1 and HA and significant negative correlation with KS in patients suffering knee OA has been observed by one of the studies (Chubinskaya *et al.* 2006). We had estimated the concentration of OP-1 in the synovial fluid of osteoarthritic patients by sandwich ELISA by using anti-OP-1(f) produced in mouse ascitic fluid developed in our laboratory whereas in the latter study done by Chubinskaya *et al.* (2006), OP-1 was estimated by using the commercially available anti-OP-1.
Though the antioxidants (SOD, GPX) are significantly lowered in blood, it did not show any significant positive/ negative correlation with the synovial fluid cartilage metabolic markers in the study. We could not find a significant correlation between OP–1 in the synovial fluid and antioxidants (SOD, GPx) in the blood. This may be due to the limitations of the study where the antioxidant assay and cartilage metabolic markers were studied in different body fluids and the total antioxidant status (TAS) assay was not done. This limitation was due to the fact that we could not get healthy volunteers for synovial fluid analysis due to ethical reasons. Further studies would be required to compare the total antioxidants in blood and synovial fluid and to correlate the findings both in serum and synovial fluid to get a true picture of the role of redox status in OA. This may be due to the fact that the assays for antioxidants were done in the blood and the cartilage markers in the synovial fluid and blood antioxidants may not truly reflect local redox status.

Comparison between use of anti-OP-1{f} developed in our laboratory and commercially available anti-OP-1 kits needs to be evaluated to further test for application in clinical diagnosis and management of knee osteoarthritis. This was not in the scope of our current study, but maybe included in our future efforts.
6.1 SUMMARY

The study was carried out in department of Biochemistry, Sikkim Manipal Institute Medical Sciences, Gangtok. A total of about 75 osteoarthritic patients attending the orthopedic department, outpatient division from CRH and STNM were selected and 75 normal, age and sex matched controls who did not suffer from OA or any other systemic disease were taken for the study.

About 5ml of heparanized venous blood were collected from osteoarthritic patients and healthy volunteers for the estimation of antioxidants like superoxide dismutase (SOD), glutathione peroxidase (GPx) and uric acid (UA). Synovial fluid samples (about 3ml) were aspirated from patients suffering from knee OA for the estimation of cartilage metabolic markers like Keratan sulfate (KS), Hyaluronic acid (HA) and Osteogenic protein-1(OP-1).

ROS are associated with the inflammatory process in OA via various pathways including the degradation of cartilage and extracellular matrix and inhibition of collagen and proteoglycan synthesis. Their levels are balanced by enzymatic and non-enzymatic antioxidant pathways for deactivation or removal of ROS. The present study observed decrease in the concentration of SOD (83.8 ± 37.80 U/ml) and GPx (2603.61±1162.7 U/l) in the blood which could be due to increase oxidative stress. The concentration of UA (4.026 ± 2.125 mg/dl) in the blood was found to be insignificant when compared with the controls. Cartilage metabolic markers like HA are responsible for its viscoelastic properties in the synovial fluid and also acts antioxidant by preventing the invasion of inflammatory cells in the joint space. The synovial fluid level of HA (19.09±11.64 ng/ml) was found to be decreased which could be due to increase of free radical degradation and decrease synthesis of
hyaluronan. While the synovial fluid level of KS was found to be increased (36.8±16.37ng/ml) when compared with the controls suggesting an increase in cartilage degradation. The study did not show any significant correlation between the antioxidants in the blood and cartilage metabolic markers in the synovial fluid of osteoarthritic patients.

Osteogenic protein-1 known as Bone morphogenetic protein-7 (BMP-7) has shown a great potential of cartilage repair and bone growth. In the present study, the OP-1 was isolated, characterized and its molecular weight was found to be around 36 kD. The purified OP-1 was immunized intraperitoneally in mouse for the production of polyclonal antibody. The anti-OP-1 collected from the ascitic fluid could detect the presence of OP-1 in the synovial fluid of osteoarthritic patients by sandwich ELISA and when compared with the reference value, the concentration was found to increased (92.8±39.67ng/ml) indicating as a part of normal reparative process. OP-1 was also detected in the blood plasma of osteoarthritic patients. The severity of OA was graded by Kellgren- Lawrence scoring system and it was found that the OP-1 level was found to be higher in grade III and IV but it was not statistically significant (p=0.09). OP-1 level in the synovial fluid was found to be correlated positively with the severity of OA. The correlation between the antioxidant enzymes (SOD & GPx), cartilage metabolic markers (HA & KS) and OP-1 was found to be insignificant (p>0.05). Whereas the correlationship between OP-1 and UA was found to be positively significant(r=0.237) suggesting that there may be a relationship between them. Significant negative correlation between OP-1 and age (r = -0.283) of osteoarthritic patients was observed which could be due to decrease in gene and protein expression of OP -1 with increasing age.
The patients between the age group of 62-67 yrs were found to be highest (32%) suffering from knee OA. Females were found to be more (70%) than males (30%) and among the ethnic groups, the Bhutias were found to be highest with knee OA (42.6%).