5.1 Introduction – The effect of glucocorticoids and PHA on Hsp60 and Hsp70 expression and release in PBMCs.

In this thesis it has been demonstrated that it is possible for Hsps to affect the OPG/RANKL system of bone regulation (Chapter 3), and that Hsps can be actively released from a range of immune cells (Chapter 4). Bone loss is seen in many situations of infection or autoimmune disease where activated immune cells would be present (section 1.10). It is known that activated immune cells can produce cytokines that affect bone loss. Activated T-cells have been shown to promote osteoclast formation in vitro (Horwood et al., 1999) (Weitzmann et al., 2001) and also to regulate bone loss in arthritis (Kong et al., 1999). It is thought that T-cells can induce osteoclast formation through RANKL dependent and independent mechanisms, and both CD4+ and CD8+ T cells have been found to stimulate osteoclast formation when activated with PHA (Weitzmann et al., 2001).

As discussed in Chapter 1 (section 1.10) many immune factors have been identified as promoters of osteoclast formation, these include RANKL, M-CSF, TNF-α, IL-1, IL-3, IL-6, IL-8. Certain immune cell cytokines such as IFN-γ have been found to negatively regulate osteoclast generation, and resting as well as activated immune cells may have an important role in the regulation of bone turnover (Shinoda et al., 2003).

Whilst there are already many identified cytokines that could be responsible for the effect of immune cells on bone turnover, it is also possible that the release of Hsps could contribute to the stimulation of bone resorption that is seen in the presence of activated immune cells in vitro and in cases of infection and autoimmunity in vivo; Hsps may also have a role in bone loss in response to certain drugs, such as corticosteroids. This chapter will aim to investigate this possibility by focusing on the following questions:

How does immune cell activation affect Hsp expression, production, and release?

Does PHA or steroid treatment up-regulate other cytokines associated with bone loss?

Could the mechanism of steroid-induced bone loss be associated with Hsp release?
5.2 Methods

5.2.1 Detection of Hsp expression in PBMCs and Whole Blood

PBMCs were isolated from whole blood (section 2.3.6) and resuspended in PBS to give a density of $2 \times 10^7$ cells/ml. The suspended cells were transferred to centrifuge tubes (1 ml/tube), and were centrifuged (3 min, 300 g) to pellet. PBS was removed from the cells and the cell pellets were resuspended in mRNA extraction buffer (section 2.3.9). The samples were either both frozen and stored at $-20\degree C$ or snap frozen in liquid nitrogen and stored at $-80\degree C$. mRNA extraction (section 2.3.9), cDNA synthesis (2.3.11) and RT-PCR (section 2.3.12) using primers for Hsp60 and Hsp90 was carried out. PCR cycle 3 was used.

5.2.2 Detection of Hsp expression in Whole Blood

mRNA extraction buffer (0.4 ml) (section 2.3.9) was added to either 40 or 80 µl of human venous whole blood. mRNA extraction (section 2.3.9), cDNA synthesis (2.3.11), and RT-PCR using primers for Hsp60 and Hsp90 was carried out. PCR cycle 4 was used (section 2.3.12).

5.2.3 Treatment of PBMCs with PHA

Prepared PBMCs (section 2.3.6) were resuspended in supplemented RPMI 1640 (2.2.13) to give a density of $\sim 2 \times 10^7$ cells/ml. The resuspended cells were pipetted into a 12 well plates (1 ml/well). Control and PHA treated samples were prepared; PHA (section 2.2.79) was added at a concentration of 4 µg/ml. After 2 hours the control and PHA treated samples were pelleted by centrifugation (3 min, 300 g), and resuspended in 1 ml supplemented RPMI 1640 media. The samples were returned to 12 well plates and were incubated at 37°C, 5% CO₂ for 3 or 24 hours. Following incubation the cells were pelleted by centrifugation (3 min, 300 g) and the pelleted cells were resuspended in 0.4 ml mRNA extraction buffer (section 2.3.9). mRNA extraction, cDNA synthesis, and RT-PCR using primers for Hsp27, Hsp60, Hsp70, Hsp90 was carried out. PCR cycle 3
was used (section 2.3.12). RANKL primers were tested using PCR cycles 3 and 4, CD 25 primers were tested using cycles 2, 3 and 4.

5.2.4 Addition of PHA, dexamethasone and prednisolone to PBMCs.

Prepared PBMCs (section 2.3.6) were washed by centrifugation, and the cell pellet was resuspended in supplemented RPMI (2.2.13) to give a density of ~ 2 x 10^7 cells/ml. The resuspended cells were pipetted into a 12 well plates (1 ml/well). The t0 control samples were immediately removed from the plate and prepared for storage (section 4.2.3). Cell treatments consisting of PHA (4 μg/ml) (section 2.2.79), Dexamethasone (100 nmol) (section 2.2.66), Prednisolone (100 nmol) (section 2.2.67), Dexamethasone (100 nmol) + PHA (4 μg/ml), and Prednisolone (100 nmol) + PHA (4 μg/ml), were added to the remaining wells, and the cells were incubated at 37°C, 5% CO₂ for 2 hours. After 2 hours the samples were washed by centrifugation (3 min, 300g x 3) and resuspended in 1 ml supplemented RPMI per sample. All treatments except PHA were re-added to the appropriate samples, and the samples were incubated at 37°C, 5% CO₂ for 8 or 24 hours. Following incubation the samples were prepared for storage and analysis (section 4.2.3). mRNA extraction (section 2.3.9) and cDNA synthesis (2.3.11) was carried out followed by RT-PCR for Hsp27, Hsp60, Hsp70, CD25, RANKL, IFN-β, and TNF-α (section 2.3.12). Media and cellular samples were analysed for Hsp60 and Hsp70 using ELISA (section 2.3.17 and 2.3.18).

5.2.5 Addition of PHA to negative T-cells

PBMCs were separated from whole blood (2.3.6) and negative T-cells were isolated using the method previously described (section 4.2.4). The T-cells were resuspended in supplemented RPMI 1640 (section 2.2.13) to give a density of 8 x 10^5/ml, and the suspended cells were then transferred to 12 well plates (1 ml/well). Treatments consisted of control or PHA treated samples; PHA was added at a concentration of 4μg/ml. After 2 hours the control and PHA treated samples were pelleted by centrifugation (3 min, 300g x 3), and resuspended in 1 ml RPMI 1640 media. The samples were returned to 12 well plates and were incubated at 37°C, 5% CO₂ for 24 hours. Following incubation the samples were prepared for storage and analysis (section 4.2.3).
5.2.6 Cell counts

Cell counts to determine cell number and viability were carried out before and after each cell treatment using the method previously described (section 4.2.3).
5.3 Results

5.3.1 Optimisation of RT-PCR to detect Hsp gene expression in PBMCs and whole blood.

Before work could be carried out on PBMC responses to activation and steroid treatments it was necessary to determine if the Hsp and RANKL primers used on bone cells would work effectively with PBMCs and blood samples. Initial work (figure 5.1) showed that Hsp expression could be detected in PBMCs under the same PCR conditions used for bone cells. There was no effect of storage temperature (figure 5.1).

RT-PCR for hsp gene expression from different quantities of whole blood was tested to determine the optimum amount of blood to be used for mRNA extraction, relatively small amounts of blood had to be used to avoid diluting the extraction buffer to a point at which it was less effective. The results showed that 80 \( \mu l \) gave the clearest results (figure 5.2).

5.3.2 Optimisation of RT-PCR to detect RANKL in PBMCs

A selection of RANKL primers were tested using cDNA obtained from control and PHA-activated PBMCs. Initial detection using 35 PCR cycles resulted in either absent or very weak bands being present; increasing the cycle number to 45 revealed that two of the primer pairs could detect RANKL expression in PBMCs (figure 5.3).

5.3.3 The effect of PHA activation on the expression of Hsp, RANKL, IFN-\( \beta \), and TNF-\( \alpha \) in PBMCs

PHA activation (24 h) caused a clear up-regulation in the expression of Hsp27, Hsp60 and Hsp70 (figure 5.4). Expression of RANKL did not show any change in response to PHA activation, and the overall level of RANKL expression was low (figure 5.5). IFN-\( \beta \) gene expression was up regulated in response to 24 h PHA activation. \( \beta \)-Actin also showed a slight increase indicating possible differences in the level of mRNA obtained from the samples.
A further experiment was carried out to look at the same responses, but at both 3 and 24 h time points. The aim was to determine if the Hsp increase was a general stress response to the treatment (it would appear at 3 h), or if it was a response to activation (24 h). Time points beyond 24 h were not used so as to avoid results being affected by increased cell number due to T-cell proliferation. The results showed that Hsp60 and Hsp70 expression was only up-regulated following 24 h PHA activation (figure 5.6). TNF-α and IFN-β were also only up-regulated at the 24 h time point. As observed previously (section 5.3.2) β-Actin showed an increase at the 24 h time point.

5.3.4 Optimisation of CD25 primers

In order to determine whether PHA was being effective in specifically activating T-cells, primers were designed to detect the T-cell specific activation marker CD25. Primers were designed to run in the same conditions as the Hsp primers. The results of the primer optimisation (figure 5.7) showed that the numbers of cycles affected how sensitively the RT-PCR allowed detection of differences in gene expression; an up-regulation of CD25 expression was seen with PHA activation, and 35 PCR cycles allowed this to be detected most clearly.

5.3.5 The effect of a 24 h treatment with PHA or a combination of PHA and dexamethasone or prednisolone on Hsp gene expression in PBMCs.

Gene expression for Hsp27 was up-regulated with 24 h PHA activation as shown previously, and it appeared to be unaffected by dexamethasone or prednisolone added alone or in combination with PHA (figure 5.8). Unlike Hsp27, Hsp60 expression was upregulated by PHA alone and by dexamethasone or prednisolone +/- PHA. Hsp70 expression was upregulated by PHA alone, but all steroid treatments caused a down-regulation in Hsp70 expression, even when added to PHA activated cells. Prednisolone had the strongest inhibitory effect on Hsp70 expression. β-Actin results for the experiment were relatively consistent with the exception of an increase in the PHA + prednisolone sample.
5.3.6 The effect of a 24 h treatment with PHA or a combination of PHA and dexamethasone or prednisolone on CD25, RANKL, IFN-β and TNF-α gene expression in PBMCs.

PHA, dexamethasone, and a combination of dexamethasone and PHA all caused a slight up-regulation in CD25 expression. Prednisolone alone appeared to have no effect, and when added in combination with PHA it reduced gene expression compared to the PHA treatment alone. TNF-α and IFN-β were both up-regulated in all PHA activated samples; this up-regulation was not reduced when the steroids were added in combination with PHA. When added alone prednisolone and dexamethasone did reduce expression of IFN-β when compared to the control (figure 5.9).

Gene expression of RANKL was inconsistent between samples. No clear up-regulation in RANKL gene expression was seen in response to PHA treatment, and expression of RANKL was either detected in the control and showed no change in response to activation, or was not detectable. Treatments that did result in a slight elevation in RANKL gene expression in PBMCs were dexamethasone and prednisolone.

5.3.7 Effect of PHA and steroids on the production and release of Hsp60 and Hsp70 in PBMCs and T-cells.

As gene expression work had shown that both PHA and steroid treatment could affect Hsp expression, it was decided to investigate if these effects would be seen at the level of Hsp protein production within the cells and if Hsp release (chapter 4) would be affected.

5.3.7.1 The effect of PHA activation and prednisolone on release and intracellular levels of Hsp60 in PBMCs at 8 and 24 h time points

An 8 h treatment with PHA (4μg/ml, 2 h, followed by 6 h incubation) resulted in a significant increase in Hsp60 release from PBMCs when compared to the 8 h control. In contrast treatment with prednisolone (figure 5.10) caused a significant reduction in Hsp60 release when compared to the 8 h control sample. When PHA and prednisolone
treatments were added in combination there was no significant difference to the 8 h control, though adding prednisolone to the PHA activated sample did cause a significant decrease in Hsp60 release compared to PHA alone. Following a 24 h incubation PHA still stimulates Hsp release, however 24 h treatment with prednisolone no longer inhibits but stimulates Hsp60 release (Figure 5.11).

Intracellular Hsp60 was significantly higher than the control following all treatments at the 8 h time point (figure 5.12). After 24 h all treatments continued to result in Hsp60 levels higher than that of the control, though PHA activated cells had Hsp60 levels of approximately 100% greater than cells treated with prednisolone alone or a combination of PHA activation and prednisolone (Figure 5.13).

5.3.7.2 The effect of PHA activation and prednisolone on release and intracellular levels of Hsp70 in PBMCs at 8 and 24 h time points

Following an 8 h incubation PHA activated cells showed no increase in Hsp70 release when compared to the 8 h control sample (figure 5.14). Prednisolone, either added alone or to the PHA activated cells caused a significant reduction (>50%) in Hsp70 release when compared to the 8 h control. At the 24 h time point prednisolone treatment continued to result in a significantly lower level of released Hsp70 when compared to the 24 h control. As with the 8 h sample, no significant difference was seen between the 24 h control and the samples treated with PHA (figure 5.15).

Intracellular Hsp70 at the 8 h time point showed no significant response to any treatment when compared to the 8 h control (figure 5.16). After 24 h intracellular Hsp70 had significantly increased in response to PHA activation (figure 5.17).

5.3.7.3 The effect of PHA on Hsp70 release and intracellular levels in T-cells.

As seen with PBMCs Hsp70 release was unchanged by PHA activation (24 h) in T-cells (figure 5.18). Intracellular Hsp70 were significantly increased following PHA activation when compared to the control (figure 5.19).
Figure 5.1. Detection of Hsp expression using RT-PCR in PBMC samples.

Lane 1. Hsp60 expression (30 PCR cycles).
Lane 2. Hsp90α expression (30 PCR cycles).
Lane 3. Hsp60 expression in PBMCs frozen in liquid nitrogen followed by -80°C storage.
Lane 4. Hsp60 expression in PBMCs frozen and stored at –20°C.
Lane 5. Hsp90α expression in PBMCs frozen in liquid nitrogen followed by -80°C storage.
Lane 6. Hsp90α expression in PBMCs frozen and stored at –20°C.
Figure 5.2. Detection of Hsp expression in whole blood samples using RT-PCR.

Lane 1. Hsp60 expression. 40 μl whole blood.
Lane 2. Hsp60 expression. 80 μl whole blood.
Lane 3. Hsp90α expression. 40 μl whole blood.
Lane 4. Hsp90α expression. 80 μl whole blood.
Figure 5.3. Testing of RANKL primers using cDNA from control and PHA activated PBMCs

Lane 1. Primer pair 1. Control.
Lane 2. Primer pair 1. PHA activated.
Lane 3. Primer pair 2. Control.
Lane 4. Primer pair 2. PHA activated.
Lane 5. Primer pair 3. Control.
Lane 6. Primer pair 3. PHA activated.
Lane 7. β-Actin. Control.
Lane 8. β-Actin. PHA activated.
Figure 5.4. The effect of PHA activation followed by a 24 h incubation on heat shock protein expression in PBMCs.

Lane 1. Hsp27 expression – Control.
Lane 2. Hsp27 expression – PHA (4μg/ml).
Lane 3. Hsp60 expression – Control.
Lane 4. Hsp60 expression – PHA (4μg/ml).
Lane 5. Hsp70 expression – Control.
Lane 6. Hsp70 expression – PHA (4μg/ml).
Lane 7. β-Actin expression – Control.
Lane 8. β-Actin expression – PHA (4μg/ml).
Figure 5.5. The effect of PHA activation followed by a 24 h incubation on RANKL and IFN-β expression in PBMCs.

Lane 1. RANKL expression – Control.
Lane 2. RANKL expression – PHA (4μg/ml).
Lane 3. IFN-β expression – Control.
Lane 4. IFN-β expression – PHA (4μg/ml).
Lane 5. β-Actin expression – Control.
Lane 6. β-Actin expression – PHA (4μg/ml).
Figure 5.6. The effect of PHA treatment of PBMCs on the gene expression of Hsp60, Hsp70, TNF-α, IFN-β, and RANKL at 3 h and 24 h time points.

Lane 1. 3 h Control.
Lane 2. 3 h PHA (4μg/ml).
Lane 3. 24 h Control.
Lane 4. 24 h PHA (4μg/ml).
Figure 5.7. Optimisation of CD25 primers: The effect of PCR cycle number on band clarity in control and PHA activated PBMC samples.

Lane 1. Control. 35 PCR cycles (section).
Lane 2. PHA (4μg/ml). 35 PCR cycles (section).
Lane 3. Control. 40 PCR cycles (section).
Lane 4. PHA (4μg/ml). 40 PCR cycles (section).
Lane 5. Control. 45 PCR cycles (section).
Lane 6. PHA (4μg/ml). 45 PCR cycles (section).
Figure 5.8. The effect of a 24h treatment with PHA, or combination of PHA and Dexamethasone or Prednisolone on the expression of Hsp27, Hsp60 and Hsp70 in PBMCs.

Lane 1. Control.
Lane 2. PHA (4 μg/ml).
Lane 3. Dexamethasone (100 nmol).
Lane 4. PHA (4 μg/ml) + Dexamethasone (100 nmol).
Lane 5. Prednisolone (100 nmol).
Lane 6. PHA (4 μg/ml) + Prednisolone (100 nmol).
Figure 5.9. The effect of a 24h treatment with PHA, or combination of PHA and Dexamethasone or Prednisolone on the expression of CD25, TNF-α, IFN-β, and RANKL in PBMCs.

1 2 3 4 5 6

CD25

TNF-α

IFN-β

RANKL

β-Actin

Lane 1. Control.
Lane 2. PHA (4 μg/ml).
Lane 3. Dexamethasone (100 nmol).
Lane 4. PHA (4 μg/ml) + Dexamethasone (100 nmol).
Lane 5. Prednisolone (100 nmol).
Lane 6. PHA (4 μg/ml) + Prednisolone (100 nmol).
Figure 5.10. The effect of PHA and Prednisolone on Hsp60 release into cell culture media from PBMCs cultured in optimal conditions for 8 h. Data presented as mean ± SEM, n = 3. Data corrected for t0 control. * p < 0.05, ** p < 0.01, *** p < 0.001 (significant difference from control).
Figure 5.11. The effect of PHA and Prednisolone on Hsp60 release into cell culture media from PBMCs cultured in optimal conditions for 24 h. Data presented as mean ± SEM, n = 3. Data corrected for t0 control. * p < 0.05, ** p < 0.01, *** p < 0.001 (significant difference from control).
Figure 5.12. The effect of PHA and Prednisolone on intracellular Hsp60 levels in PBMCs cultured in optimal conditions for 8 h. Data presented as mean ± SEM, n = 3.

* p < 0.05, ** p < 0.01, *** p < 0.001 (significant difference from control).
Figure 5.13. The effect of PHA and Prednisolone on intracellular Hsp60 levels in PBMCs cultured in optimal conditions for 24 h. Data presented as mean ± SEM, n = 3. * p < 0.05, ** p < 0.01, *** p < 0.001 (significant difference from control).
Figure 5.14. The effect of PHA and Prednisolone on Hsp70 release into cell culture media from PBMCs cultured in optimal conditions for 8 h. Data presented as mean ± SEM, n = 3. Data corrected for t0 control.  

* p < 0.05, ** p < 0.01, *** p < 0.001 (significant difference from control).
Figure 5.15. The effect of PHA and Prednisolone on Hsp70 release into cell culture media from PBMCs cultured in optimal conditions for 24 h. Data presented as mean ± SEM, n = 3. Data corrected for t0 control. * p < 0.05, ** p < 0.01, *** p < 0.001 (significant difference from control).
Figure 5.16. The effect of PHA and Prednisolone on intracellular Hsp70 levels in PBMCs cultured in optimal conditions for 8 h. Data presented as mean ± SEM, n = 3.

* p < 0.05, ** p < 0.01, *** p < 0.001 (significant difference from control).
Figure 5.17. The effect of PHA and Prednisolone on intracellular Hsp70 levels in PBMCs cultured in optimal conditions for 24 h. Data presented as mean ± SEM, n = 3.

* p < 0.05, ** p < 0.01, *** p < 0.001 (significant difference from control).
Figure 5.18. Hsp70 release into media from isolated negative T-cells following a 24 h incubation after initial PHA treatment (4μg/ml, 2 h). Data presented as mean ± SEM, n = 3. Data corrected for t0 control. * p < 0.05, ** p < 0.01, *** p < 0.001 (significant difference from control).
Figure 5.19. Intracellular Hsp70 levels in isolated negative T-cells following a 24 h incubation after initial PHA treatment (4μg/ml, 2 h). Data presented as mean ± SEM, n = 3. * p <0.05, ** p <0.01, *** p <0.001 (significant difference from control).
5.4 Discussion

Hsp gene expression at the 24 h time point was clearly up regulated by PHA activation in all experiments, though β-Actin also showed an increase in two out of four cases. This effect of Hsp increase in response to PHA was also seen in the intracellular levels following 24 h incubation in PBMCs and T-cells. Gene expression of Hsps following 3 h incubation with PHA did not show increased expression, suggesting that the response seen later was due to activation rather than stress caused by the treatment itself. Hsp release showed slightly different patterns to intracellular responses with only Hsp60 release showing an increase in response to PHA treatment. The release of Hsp60 in response to activation suggests the potential exists for bone cells to be affected directly. Even if this is not the case it is possible that the increase of Hsp expression and production in response to activation within the cell may not act directly on bone, but could in turn lead to increased production of cytokines that would influence osteoclast formation.

Prednisolone clearly inhibited Hsp60 and Hsp70 release at the 8 h time point when added alone and in combination with PHA, however at the 24 h time point this effect disappeared, with the exception of prednisolone and Hsp70. This change in Hsp release patterns at 24 h could be attributed to the fact that CD8⁺ lymphocytes are sensitive to dexamethasone induced apoptosis (Marchetti et al., 2003) and whilst the cell viability staining did not show a significant difference between steroid treated samples and the control, it is possible that steroid induced apoptosis may have been responsible for this effect at 24 h.

RANKL has been previously reported to be expressed in activated T-cells and is known to be involved in immune cell regulation (Wong et al., 1997) (Bachmann et al., 1999) (Josien et al., 1999). It has been concluded that RANKL production by activated T-cells is responsible for bone loss and joint damage in adjuvant arthritis (Kong et al., 1999). In the work carried out in this chapter the background expression and responses of RANKL were inconsistent compared to those of the Hsps and other cytokines, particularly in blood cells obtained from different individuals. No clear up-regulation in RANKL gene expression was seen in response to PHA treatment, and gene expression was either detected and showed no change in response to activation, or was not detectable. The only treatments that resulted in a slight elevation in RANKL gene
expression in PBMCs were dexamethasone and prednisolone, and whilst further work would need to be carried out to determine which immune cell types were involved, it may have implications in the explanation of steroid induced bone loss.

As expected, TNF-α expression was upregulated when compared to the control 24 h after PHA treatment. TNF-α is a known stimulator of bone resorption (Gorny et al., 2004) that has been implicated in the development of arthritis related bone erosion (Ritchlin et al., 2003) and oestrogen deficiency related bone loss (Censi et al., 2000); It has been shown that a treatment consisting of anti-TNF-α combined with OPG can reverse arthritis-related bone loss in mice (Redlich et al., 2004). While it is possible TNF-α could have a role in bone loss associated with immune cell activation, glucocorticoids had no effect on TNF-α expression either alone or in PHA-activated cells. This suggests that TNF-α production by immune cells is not related to glucocorticoid-induced bone loss.

IFN-β was upregulated in the PHA-activated samples by the 24 h time point. When added in combination with PHA, prednisolone or dexamethasone did not cause a change in IFN-β expression, yet when added alone the steroids did appear to reduce expression of IFN-β when compared to the control. It has been found that IFN-β can inhibit the differentiation of osteoclast precursor cells by interfering with RANKL-induced expression of c-Fos, which is an essential transcription factor for osteoclast formation (Takayanagi et al., 2002), and that IFN-β expression is induced by RANKL (Takayanagi et al., 2002), (Hayashi et al., 2002). Though these studies were carried out using osteoclast precursors only, it is possible that IFN-β produced by immune cells could contribute to this effect in vivo. This is supported by the fact that it has been demonstrated that T-cell production of IFN-γ suppressed osteoclastogenesis by interfering with the RANKL/RANK signalling pathway (Takayanagi et al., 2000). These findings suggest it is possible that the inhibition of IFN-β by steroids in non-activated PBMCs may have relevance to steroid-induced bone loss, and it would be an interesting area for further study.

Overall the results of this chapter suggest that it may be possible for Hsps that are produced and/or released from activated immune cells to influence bone turnover,
though many questions remain as to whether they would have a direct influence, or act through the stimulation or inhibition of other bone influencing cytokines. Generally glucocorticoid treatment had an inhibitory effect on Hsp release, suggesting that Hsps were not linked to steroid-induced bone loss, though the potential apoptosis inducing effect of steroids may have implications in terms of an increase of Hsps due to cell damage. Gene expression studies did reveal one factor that may be involved in steroid induced bone loss; the down regulation of IFN-β.