Chapter 3: Heat Shock Protein Localisation in Chronic Lymphocytic Leukaemia

3.1 Introduction

This chapter investigates the localisation of Hsp72, Hsp90 and Hsp27 in Chronic Lymphocytic Leukaemia (CLL) to determine whether expression of these stress proteins has any clinical or prognostic relevance.

3.1.1 Chronic Lymphocytic Leukaemia (CLL)

Chronic Lymphocytic Leukaemia (CLL) is the most common haematological malignancy in adults (Yee & O'Brien 2006). The disease is characterised by clonal expansion of B-cells with the immunophenotype CD5+/CD19+/CD23+/sIg^low/CD79b^low/FMC7^. Accumulation of these cells occurs within the blood, bone marrow, and secondary lymphoid organs (Falt et al. 2005). Approximately 50% of patients are asymptomatic at time of diagnosis but the condition may progress to a symptomatic form requiring treatment. Median age at diagnosis is approximately 70 years and so CLL can be considered to be an age-related disease.

CLL may be classified either by the Binet classification system or the Rai classification system, although the former is used by the majority of physicians. The Binet system is divided into three subtypes based upon the presence of lymphadenopathy, splenomegaly, anaemia and thrombocytopenia (Table 3.3.1).

Table 3.3.1: The subtypes of CLL according to the Binet classification

<table>
<thead>
<tr>
<th></th>
<th>Description</th>
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<tbody>
<tr>
<td>A</td>
<td>Fewer than 3 areas of lymphoid tissue are enlarged, with no anaemia or thrombocytopenia</td>
</tr>
<tr>
<td>B</td>
<td>3 or more areas of lymphoid tissue are enlarged, with no anaemia or thrombocytopenia.</td>
</tr>
<tr>
<td>C</td>
<td>Anaemia and thrombocytopenia</td>
</tr>
</tbody>
</table>
3.1.2 Prognostic Markers in CLL

CLL is a heterogeneous disease with a variable clinical course. Therefore, numerous studies have been conducted to identify prognostic markers that may aid in predicting clinical outcome.

The mutation status of the immunoglobulin variable heavy chain genes (IgV<sub>H</sub>) is now routinely analysed, as lack of mutations (IgV<sub>H low</sub>) identifies a more immature disease and correlates with worse prognosis, while IgV<sub>H high</sub> represents a more mature disease with better prognosis (Hamblin, 2007; Khartan-Dabaja et al. 2008).

Expression of CD38 on B-CLL cells has been shown to correlate with poor response to treatment and shorter overall survival (Hus et al. 2006; Deaglio et al. 2006). This surface molecule is found on various haematopoetic cells and functions in the regulation of cell activation and proliferation (Cruse et al. 2007). It has been hypothesised that the expression of CD38 on CLL cells aids in the continued proliferation and survival of these cells (Deaglio et al. 2006).

Expression of the tyrosine kinase Zeta-associated Protein-70 (ZAPE70) has also been shown to correlate with poor prognosis. In normal T-cells, ZAP-70 is associated with the TCR where it functions in downstream TCR signalling (Cruse et al. 2007). However, although ZAP-70 is present in normal pre-B-cells, its expression should be lost on maturation of the cell. Hence, the presence of ZAP-70 in CLL cells is indicative of an immature clone. Studies have shown a correlation between un-mutated IgV<sub>H</sub> and ZAP-70 expression (Orchard et al. 2004; Crespo et al. 2003; Chen et al. 2002b). Moreover, patients positive for ZAP-70 have been shown to respond poorly to treatment with purine analogues. In view of this, ZAP-70 expression is widely analysed in newly diagnosed CLL.

Cytogenetic analysis of CLL patients can give an early indication of disease severity with some genetic defects correlating strongly with poor response to treatment and shorter overall survival. Fluorescent in-situ Hybridisation (FISH) techniques are used to identify the underlying genetic mutations that have occurred in B-CLL cells. There are currently four main genetic defects recognised in CLL patients (Table 3.3.2).
Table 3.3.2: Genetic defects found in CLL cells and their associated prognoses. (adapted from Cotter & Auer, (2007) and Van Bockstaele et al. (2009)).

<table>
<thead>
<tr>
<th>Defect</th>
<th>% of CLL cases</th>
<th>Gene Affected</th>
<th>Median OS* (months)</th>
<th>Median TFS* (months)</th>
<th>Prognosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trisomy 12</td>
<td>15-30</td>
<td>CDK4, CDKN1B, CLLU1, MDM2</td>
<td>114</td>
<td>33</td>
<td>Intermediate</td>
</tr>
<tr>
<td>Del 17p</td>
<td>10</td>
<td>TP53</td>
<td>32</td>
<td>9</td>
<td>Poor</td>
</tr>
<tr>
<td>Del 11q</td>
<td>15-20</td>
<td>ATM, ARHGAP20, BTG4, POU2AF1, FDX1, RDX</td>
<td>79</td>
<td>13</td>
<td>Poor</td>
</tr>
<tr>
<td>Del 13q</td>
<td>40-60</td>
<td>ARL11, C13orf1, DLEU1, DLEU2, RCBTB1, SETDB2, TRIM13</td>
<td>133</td>
<td>92</td>
<td>Good</td>
</tr>
</tbody>
</table>

*OS – Overall Survival
**TFS – Treatment-free Survival

CLL patients presenting with a 17p deletion demonstrate a fundamentally different clinical course and therefore studies into p53 defects and alternative treatment regimes have become more common. Mutations of the TP53 gene are displayed by 4-37% of patients with CLL (Zenz et al. 2008). Furthermore, patients resistant to fludarabine treatment show the highest incidence of TP53 mutation. Agents that act independently of the p53 pathway, such as the monoclonal antibodies rituximab and alemtuzumab and other agents such as lenalidomide, have become available and may be used as an alternative first-line treatment for patients with a 17p deletion.

3.1.3 Treatment of CLL

Due to the considerable variation in disease progression, management of CLL is problematic. In the majority of cases, CLL progresses slowly and patients may go for many years without requiring treatment. The decision to start treatment is usually taken when blood counts start to fall or quality of life becomes considerably affected. A platelet count of <100 and a haemoglobin level of <10 is considered a good indicator to start treatment.
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Chemotherapeutic treatment of CLL usually involves alkylating agents, purine analogues, anthracyclines or combinations of these drugs. Alkylating agents such as cyclophosphamide exert their effects by replacing a hydrogen atom in DNA with an alkyl radical. This prevents correct DNA base-pairing and the DNA becomes fragmented. Purine analogues such as fludarabine interfere with ribonucleotide reductase and DNA polymerase thereby inhibiting DNA synthesis. The drug is transported into CLL cells where it is converted into its 5’-triphosphate, the primary active metabolite (Plunkett, Huang & Gandhi, 1990). It may then compete for incorporation into the A sites of elongating DNA by DNA polymerase, thus terminating DNA synthesis and resulting in cell death. Anthracyclines such as doxorubicin form DNA adducts preventing DNA replication. Combination-based therapies involving fludarabine and cyclophosphamide have been shown to be more effective than using these drugs in isolation. Frequently cancer cells are able to overcome the alkylating agent-induced DNA damage by DNA repair mechanisms. However, by treating in conjunction with fludarabine, this purine analogue can inhibit the repair of alkylating-agent induced DNA interstrand crosslinks (Nabhan et al. 2008). As a result, fludarabine and cyclophosphamide combination treatment is the most common first-line treatment of CLL.

Patients who fail to respond to first-line treatment are often treated with a combination of cyclophosphamide, doxorubicin, the mitotic inhibitor vincristine and the corticosteroid prednisone (CHOP). However, only approximately 40% of patients resistant to fludarabine will be expected to survive beyond 12 months (Keating et al. 2002).

The vast majority of patients with a 17p deletion abnormality will fail to respond to conventional first-line treatments and therefore are often treated with monoclonal antibodies such as rituximab or alemtuzumab directed against the B-cell antigens CD20 and CD52 respectively. These antibodies exert their effects via antibody-dependent cellular cytotoxicity (ADCC) and apoptosis (Kim et al. 2007). As they are acting independently of the p53 pathway, rituximab and alemtuzumab have shown promising results as a first-line treatment in 17p deletion patients. Additionally, alemtuzumab in combination with fludarabine has shown promising results in patients found to be unresponsive to these treatments in isolation (Kennedy et al. 2002).
3.1.4 CLL and Autoimmune Haemolytic Anaemia (AIHA)

The clinical course of patients with CLL is often exacerbated by autoimmune destruction of erythrocytes. When erythropoesis can no longer match haemolysis, the symptoms of haemolytic anaemia present. Although AIHA can occur in early stage CLL patients, it is most common in patients with advanced disease (Diehl & Ketchum, 1998). A number of mechanisms have been proposed to explain the development of AIHA in CLL patients. One suggestion is that as T-cell function is impaired in CLL patients, autoimmunity maybe due to loss of T-cell regulatory control of autoreactive T-cells (D’arena et al. 2007). Moreover, it has been demonstrated that T regulatory cells are sensitive to fludarabine (Beyer et al. 2005) and as AIHA is more prevalent in advanced CLL, it would seem more likely that these patients will have received fludarabine treatment. Other evidence has suggested that CLL cells act as major APCs and process and present Rhesus antigen to stimulate T-helper cells (Hall et al. 2005). This ultimately leads to a T-cell response mounted against erythrocytes.

Corticosteroids are usually the first line treatment of AIHA in CLL patients. Prednisolone at a dose of 1.0-1.5mg/kg/day typically results in 80% initial response (D’arena et al. 2007). However, only a minority of patients are able to achieve a long lasting remission and therefore require a lower dose of long-term steroid treatment. Patients resistant to the effects of corticosteroids may be treated with rituximab and alemtuzumab. The consequent ADCC results in a reduction in the number of antigen-presenting B-cells, with a decrease on T-cell activation (D’arena et al. 2007). Splenectomy may be considered for patients unresponsive to other lines of treatment.

3.1.5 Immunodeficiency in CLL

The large majority of CLL patients possess a level of immunodeficiency and the presence of CLL cells in the lymphoid tissues interferes with nearly all facets of immune function. Hypogammaglobulinaemia, T-cell depletion and reduced T-cell function are the most common presentations (Hamblin et al. 2008). The degree of hypogammaglobulinaemia is dependent upon the stage of disease but is independent of whether the patient has received chemotherapeutic treatment. As the number of
non-malignant B-cells is reduced, B-cell function is reduced as normal immunoglobulin production is suppressed. The increased number of T regulatory cells in CLL is also thought likely to contribute to immunodeficiency. Often treatment of CLL, especially by fludarabine, exacerbates the immunodeficiency as T-helper (T_h) cell levels fall and remain low for up to two years after treatment (Hamblin et al. 2008).

3.1.6 Heat Shock Protein Expression in CLL

A large amount of research into both the expression and subsequent function of HSPs in the development of CLL is still necessary. A small number of studies has been performed investigating the expression of HSPs in myeloid malignancies, and results from such studies might be further interpreted to provide a predictive analysis of HSP function in CLL. However, the huge clinical variability in CLL compared to AML may signify that HSP expression varies greatly between leukaemias making such predictions invalid.

Research into acute lymphoblastic leukaemia (ALL) has shown decreased expression of Hsp72 and Hsp27 in bone marrow aspirates from patients who achieved complete remission when compared to those patients who did not achieve complete remission (Campos et al. 1999). Additionally, ALL cells displaying the Bcr/Abl fusion protein contained high levels of Hsp72 (Nimmanapalli et al. 2002). Further studies have demonstrated that Hsp72 contributes to the Bcr-Abl–mediated resistance to apoptosis by chemotherapeutic agents such as etoposide. Moreover, down-regulation of Hsp72 can sensitise these Bcr/Abl ALL cells to cytotoxic drugs (Guo et al. 2005). The Bcr/Abl fusion protein was shown to be a client protein of Hsp90. Furthermore, inhibition of Hsp90, but not Hsp72, in myeloid cells was found to result in degradation of Bcr/Abl (Peng et al. 2007).

Treatment of ATM/TP53 mutated CLL cells with the Hsp90 inhibitor, GA caused a down-regulation of mutant p53 and a simultaneous up-regulation of wild type p53. GA treatment also resulted in an increase in p21, an inducer of cell cycle arrest (Lin et al. 2007b). Further research has shown a synergistic effect between a derivative of
GA, 17-DMAG and Dox, demonstrating a sensitisation of p53 mutated cells to Dox-induced cell death (Robles et al. 2006).

As previously mentioned, expression of ZAP-70 is a predictor of poor prognosis in CLL. Furthermore this tyrosine kinase has been shown to be a conditional Hsp90 client protein (Bartis et al. 2007). Castro et al. (2005), demonstrated that ZAP-70 in CLL cells co-immunoprecipitates with Hsp90, while ZAP-70 from normal T-cells does not. In addition, treatment of CLL cells with Hsp90 inhibitors resulted in degradation of ZAP-70, while treatment of T-cells from CLL patients and control subjects with Hsp90 inhibitors did not affect ZAP-70.

Taken together, the results presented so far show that HSPs can be over-expressed in leukaemias of lymphoid origin. Inhibition of these HSPs by various compounds results in degradation of client proteins and ultimately cell death. However, the location of HSPs, whether expressed intracellularly or on the cell surface, in CLL cells has not been investigated. Additionally, comparisons between HSP expression in CLL patients and control subjects have not yet been drawn.

3.1.7 Aims

The multistep progression to carcinogenesis may take several decades and therefore an increasingly ageing population allows age-related leukaemias to become more frequent. The difficulties involved in treating these patients and the frequent occurrence of drug resistance warrant investigation into the possible mechanisms involved in CLL progression. Therefore, the aim of this chapter is to explore both the internal and surface localisation of Hsp72, Hsp90 and Hsp27 in CLL patients and age-matched control subjects to determine if levels of these stress proteins have any clinical or prognostic connotations.
3.2 Methods

Local research ethics committee approval (COREC - 05/Q1506/103) was obtained for this study and consent forms were completed by each patient. Blood samples were collected from patients affected by CLL (n= 40). Blood from normal age matched control subjects (n=9) was also obtained. Blood was collected by venepuncture in 7ml EDTA tubes or 7ml Serum tubes (Southern Syringe Services).

The localisation of HSPs was determined by flow cytometry and western blotting. Surface and intracellular Hsp27, Hsp90 and Hsp72 were analysed in total leukocytes isolated from lysed whole blood (see section 2.2.3). T Regulatory cell numbers and levels of caspase-3 were also analysed by flow cytometry. Release of Hsp72 into serum was analysed by ELISA.

3.2.1 Total Leukocyte Isolation
Leukocytes from whole blood were isolated using methods described in section 2.2.1.1

3.2.2 PBMC Purification
PBMCs were isolated from whole blood using methods described in section 2.2.1.2

3.2.3 Caspase-3 Analysis
Caspase-3 analysis was performed on leukocytes from CLL patients and control subjects, to determine the levels of apoptosis in these patients. Caspase-3 analysis was performed according to the methods described in section 2.2.3.2.

3.2.4 Surface HSP Analysis
Surface HSP analysis was performed on leukocytes from CLL patients and control subjects according to the methods described in section 2.2.3.4

3.2.5 Intracellular HSP Analysis
Intracellular HSP analysis was performed on leukocytes from CLL patients and control subjects according to the methods described in section 2.2.3.5
3.2.6 ZAP-70 Analysis

ZAP-70 analysis was performed on leukocytes from CLL as prognostic indicator. Analysis was performed according to the methods described in section 2.2.3.6

3.2.7 T-Regulatory Cell Detection

Treg analysis was performed on PBMCs from CLL patients and control subjects according to the methods described in section 2.2.3.7

3.2.8 Western Blot Analysis

Western blot analysis was performed on cell extracts from PBMCs to confirm the presence of Hsp72 in CLL patients. Analysis was performed according to the methods described in section 2.2.4

3.2.9 Hsp72 ELISA

Hsp72 ELISAs were performed on serum from CLL patients and control subjects. ELISAs were performed according to the methods described in section 2.2.5.

3.2.10 Statistical Analysis

Statistical analysis was performed using the t-test or one-way-ANOVA with Dunnet’s or Bonferroni’s post-hoc tests depending on the data analysed: P values < 0.05 were considered to be significant. Where the t-test was used and significant differences were found, specific p values are stated. Where the one-way ANOVA was used and significant differences were found, approximate p values are stated. * represents a p value < 0.05, ** represents a p value < 0.01 and *** represents a p value < 0.001. Correlation analysis was performed using the Spearman’s correlation coefficient; P values < 0.05 were considered to be significant. Where significant differences were found, the precise p value and r value are stated.
3.3 Results

The characteristics of the patients involved in this study can be seen in table 3.3.3.

Table 3.3.3: Characteristics of the CLL patients and control subjects involved in this section of the study. Data is represented as mean (range).

<table>
<thead>
<tr>
<th></th>
<th>CLL Patients</th>
<th>Control Subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients included</td>
<td>40</td>
<td>14</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>28/12</td>
<td>6/8</td>
</tr>
<tr>
<td>Mean Age of Patients (Range)</td>
<td>66 (45-80)</td>
<td>66(42-88)</td>
</tr>
<tr>
<td>White blood cell count at time of sample (x10^9/L)</td>
<td>66.99 (1.2-326.5)</td>
<td>6.87(3.7-10.3)</td>
</tr>
<tr>
<td>Haemoglobin at time of sample (g/dL)</td>
<td>12.62 (8-15.3)</td>
<td>13.1(10-14.4)</td>
</tr>
<tr>
<td>Platelet count at time of sample (x10^9/L)</td>
<td>183.03 (33-381)</td>
<td>257.16(128-402)</td>
</tr>
</tbody>
</table>

3.3.1 Active Caspase-3 Activity in Chronic Lymphocytic Leukaemia

Expression of active caspase-3 in total lymphocytes of CLL patients was compared with the expression in lymphocytes from control subjects to determine if there was any difference in the degree of apoptosis between the two patient groups.

Patient data was grouped according to disease and then compared for the expression of active-caspase-3. As shown in Figure 3.3.1, CLL patients showed a lower expression of active caspase-3 in total lymphocytes than control subjects (p<0.0008). When CLL patients were grouped according to Binet stage, no significant difference in active caspase-3 was found between stages. However patients in all three Binet stages showed caspase-3 levels lower than levels observed in control subjects (Figure 3.3.2).
Figure 3.3.1: Active Caspase-3 expression in CLL patients and control subjects.
Active caspase-3 expression in total lymphocytes (CD5+/CD19+ and CD5-/CD19+ cells) from CLL patients and lymphocytes (gated using FSC/SSC) from control subjects was analysed by flow cytometry. Each group of patients is represented as a Mean of the MFI values, +/- SEM. Statistical Analysis was performed using the unpaired T-test.

Figure 3.3.2: Active Caspase-3 expression in CLL patients grouped according to Binet stage.
Active caspase-3 expression in total lymphocytes (CD5+/CD19+ and CD5-/CD19+ cells) from CLL patients and lymphocytes (gated using FSC/SSC) from control subjects was analysed by flow cytometry. Each group of patients is represented as a Mean of the MFI values, +/- SEM. Statistical Analysis was performed using the one-way ANOVA with Bonferroni’s post hoc test. * represents a p value < 0.05. ** represents a p value < 0.01.
3.3.2 Surface and Intracellular Heat Shock Protein 72 Detection.

Localisation of surface Hsp72 (sHsp72) and intracellular Hsp72 (iHsp72) was analysed on the leukocytes of CLL patients and control subjects. For surface Hsp72 analysis, the cmHsp70.1 antibody (Multimmune Ltd) was used. This antibody recognises the TKD (TKDNNLLGRFELSG) peptide region of the Hsp72 protein, a region shown to be presented outside the cell surface when the protein is embedded in the cell surface. For intracellular analysis, the mouse monoclonal anti-human Hsp72 antibody (Stressgen) was used. This antibody cannot recognise surface embedded Hsp72, as its epitope remains hidden in the cell membrane. However, receptor attached Hsp72 that is bound loosely to the cell surface can be detected by the mouse monoclonal anti-human Hsp72 antibody (Stressgen). The difference in signal detected when comparing surface Hsp72 analysis using the cmHsp70.1 antibody and the mouse monoclonal anti-human Hsp72 antibody can be seen clearly when observing the flow cytometry histograms. Figure 3.3.3 illustrates the increased signal detection when analysing surface Hsp72 expression in a control subjects using the cmHsp70.1 antibody (Figure 3.3.1B) and the anti-Hsp72 antibody (Figure 3.3.3C). Experiments involving treatment of control leukocytes with different concentrations of sodium chloride demonstrated a decreased antibody recognition when analysing sHsp72 with the mouse monoclonal anti-human Hsp72 antibody (Table 3.3.4). This indicates that the sHsp72 being detected by this antibody can in fact be ‘washed off’ and therefore is thought to be only loosely bound to the cell surface.
Figure 3.3.3: Comparison of surface Hsp72 localisation analysed using the cmHsp70.1 and the Stressgen antibody.

Hsp72 localisation on a representative normal control subject was analysed by flow cytometry: neutrophils (blue), monocytes (green) and lymphocytes (red) were gated using FSC/SSC.  

A. sHsp72 expression on leukocytes from a control subject analysed using the cmHsp70.1 antibody  

B. sHsp72 on leukocytes from a control subject analysed using the Stressgen antibody  

C. Isotype control for sHsp72.
Table 3.3.4: Effect of sodium chloride treatment on sHsp72 expression analysed using the Stressgen antibody.

sHsp72 expression was analysed on the leukocytes of a control subject after treatment with different concentrations of NaCl. Non-viable cells were eliminated from the analysis by gating on propidium iodide-negative cells. Neutrophils, monocytes and lymphocytes were gated using FSC/SSC.

<table>
<thead>
<tr>
<th>Concentration of NaCl (mM)</th>
<th>sHsp72 Mean MFI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Neutrophils</td>
</tr>
<tr>
<td>0</td>
<td>565</td>
</tr>
<tr>
<td>20</td>
<td>357</td>
</tr>
<tr>
<td>40</td>
<td>287</td>
</tr>
<tr>
<td>No Stain control</td>
<td>117</td>
</tr>
</tbody>
</table>
3.3.3 Heat Shock Protein 72 Localisation in Chronic Lymphocytic Leukaemia

In samples from control subjects, neutrophil, monocyte and lymphocyte populations were distinguished using FSC/SSC. In samples from CLL patients, neutrophil, monocyte and total lymphocyte populations were all distinguished using FSC/SSC, while malignant and non-malignant cells were gated using CD5+/CD19+ (Figure 3.3.4) and CD5-/CD19+.

Patient data was grouped according to CLL or control and then compared for the expression of both sHsp72 and iHsp72. When sHsp72 expression on CD5+/CD19+ cells in CLL patients group as a whole, was compared with sHsp72 expression on normal lymphocytes from control subjects, there appeared to be no significant difference in expression of either sHsp72 or iHsp72 between the groups. However, the spread of data within the CLL group was very large and further analysis revealed that some CLL patients express a very high level of sHsp72 on their CD5+/CD19+ cells, while some patients express a very low level (Figure 3.3.5). A similar pattern was observed with iHsp72 in CLL patients. This was not the case when observing expression of sHsp72 or iHsp72 on CD5-/CD19+ cells from CLL patients or CD19+ cells from control subjects. This difference in sHsp72 and iHsp72 expression among CLL patients was also prominent on the flow cytometry histograms (Figure 3.3.6).

CLL patients were then divided into 2 distinct groups dependent upon the expression of sHsp72 on their CD5+/CD19+ cells. The cut off value chosen to divide the two groups was the mean sHsp72 expressed by control subjects. Expression of sHsp72 on CD5+/CD19+ cells in these high- and low-expressing patient groups was then compared to sHsp72 expression in both CD5-/CD19+ cells from the same CLL patients and lymphocytes from control subjects (Figure 3.3.7). The high sHsp72 expressing group was significantly different (100 fold higher) from the low expressing group (P<0.05), the CD5+/CD19+ lymphocytes (P<0.05) and the lymphocytes from control subjects (P<0.01). The low expressing sHsp72 group was not significantly different either from the CD5+/CD19+ lymphocytes or the lymphocytes from control subjects (Figure 3.3.7).
Comparison of iHsp72 expression in CD5+/CD19+ cells with expression in CD5-/CD19+ cells and normal lymphocytes from control subjects presented a similar pattern (Figure 3.3.8): The high iHsp72 group was significantly different from the low expressing CD5+/CD19+ group (P<0.001) and from CD5+/CD19+ lymphocytes (P<0.001) and control lymphocytes (P<0.001). However, the low expressing intracellular Hsp72 group was not significantly different either from the CD5+/CD19+ lymphocytes or the lymphocytes from control subjects. A western blot analysis was performed on PBMCs from two representative CLL patients categorised as being high- or low-expressers of iHsp72 (Figure 3.3.9). This confirmed results obtained by flow cytometry, by showing that there are significant differences in iHsp72 expression between patients with CLL. It is important to note that no correlation was found between expression of sHsp72 and iHsp72 in CD5+/CD19+ cells and therefore patients expressing high sHsp72 did not necessarily express high iHsp72 (data not shown).

An attempt was made to draw correlations between the expression of sHsp72 in CLL patients and stage of disease. However, when grouped according to Binet stage, only two patients analysed for sHsp72 expression were classed as Binet stage B and so drawing accurate conclusions proved difficult. Figure 3.3.10 illustrates the range of expression of sHsp72 in all three Binet stages. Both early and advanced stage patients appeared to display comparable levels of expression. When iHsp72 expression in CLL patients was analysed in this way, a similar result was observed.

Surface and intracellular Hsp72 expression in CD5+/CD19+ cells was compared in CLL patients who had stable disease (not requiring treatment) and CLL patients who had progressive disease (requiring treatment). Stable and progressive disease patients express very similar levels of sHsp72 (data not shown). However, when the expression of iHsp72 was analysed in this way, patients with stable disease were found to express significantly higher levels of iHsp72 than patients with progressive disease (P=0.0138) (Figure 3.3.11).

Attempts were made to draw correlations between the expression of both surface and intracellular Hsp72 with levels of caspase-3 in CD5+/CD19+ cells, however, no associations could be found (data not shown).
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Figure 3.4.4: Identification of Malignant CLL cells
Gating of the malignant cell population in CLL patients was achieved using an anti-human CD5-APC/anti-human CD19-PE double staining method.

Figure 3.3.5: Surface and Intracellular Hsp72 expression on the malignant cells from CLL patients.
sHsp72 and iHsp72 was analysed by flow cytometry. CD5+/CD19+ malignant cells were gated. Values are expressed as absolute MFI and plotted in log scale. Surface Hsp72 analysis was performed on 17 CLL patients, while intracellular Hsp72 analysis was performed in 40 CLL patients. Boxes highlight the distinct groups of patients expressing significantly different levels of Hsp72.
Figure 3.3.6: Surface and Intracellular Hsp72 localisation in representative CLL patients.

Samples were analysed by flow cytometry: B-CLL cells (blue) and lymphocytes (green) were gated using CD5+/CD19+ and CD5-/CD19+ respectively. (A) High surface-Hsp72 expressing patient (B) Low sHsp72 expressing patient (C) High iHsp72 expressing patient (D) Low iHsp72 expressing patient.
Figure 3.3.7: Comparison of sHsp72 in high- and low-expressing patients.
Surface Hsp72 expression on the malignant and normal B-lymphocytes from CLL patients and lymphocytes from age-matched control subjects was analysed by flow cytometry. CD5+/CD19+ malignant cells and CD5-/CD19+ normal B cells were gated. Each group of patients is represented as a Mean of the MFI values, +/- SEM and plotted on a log scale. Statistical analysis was performed using the 1 way ANOVA with Bonferroni’s multiple comparison test. * represents a p value < 0.05. ** represents a p value < 0.01.
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Figure 3.3.8: Comparison of iHsp72 in high- and low-expressing patients. Intracellular Hsp72 expression on the malignant and normal B-lymphocytes from CLL patients and lymphocytes from age-matched control subjects was analysed by Flow Cytometry. CD5+/CD19+ cells and CD5-/CD19+ cells were gated. Each group of patients is represented as a Mean of the MFI values, +/- SEM and plotted on a log scale. Statistical analysis was performed using the 1 way-ANOVA with Bonferroni’s multiple comparison test.

Figure 3.3.9: Western Blot of intracellular Hsp72 expression in high- and low-expressing CLL patients. Cell extracts were prepared from PBMCs purified from whole blood. Data is representative of 22 high-expressing patients and 18 low-expressing patients.
Figure 3.3.10: Surface and Intracellular Hsp72 in CLL patients grouped according to Binet stage.
(A) sHsp72 and (B) iHsp72 expression in CD5+/CD19+ cells from CLL patients were analysed by flow cytometry. Data is plotted on a log scale.
**Figure 3.3.11: Comparison of intracellular Hsp72 expression in CLL patients with stable and progressive disease.**

iHsp72 in total lymphocytes from CLL patients was analysed by Flow Cytometry. Each group of patients is represented as a Mean of the MFI values, +/- SEM. Statistical analysis was performed using the unpaired t-test.
3.3.3.1 Release of Hsp72 into Serum

Hsp72 was analysed in serum samples from CLL patients and control subjects by ELISA. There was no significant difference in levels of extracellular Hsp72 between CLL patients and control subjects (Figure 3.3.12). However, further examination revealed that CLL patients receiving corticosteroid treatment were releasing significantly lower levels of Hsp72 than patients not receiving corticosteroid treatment (Figure 3.3.13). Furthermore, analysing release of Hsp72 in individual patients at different time points revealed that on commencement of corticosteroid treatment, Hsp72 levels in serum dramatically decrease and remain low for the duration of the treatment (Figure 3.3.14). Patients show approximately a 70% reduction in Hsp72 release one month after initiation of corticosteroid treatment.
Figure 3.3.12: Extracellular Hsp72 in serum from CLL and control subjects.
Extracellular Hsp72 in serum from CLL patients and control subjects was analysed by ELISA. Data is plotted on a log scale.

Figure 3.3.13: Comparison of Extracellular Hsp72 in CLL patients treated with or not treated with corticosteroids.
Extracellular Hsp72 in serum from CLL patients and control subjects was analysed by ELISA. Statistical analysis was performed using the one-way ANOVA with Bonferroni’s multiple comparison post-hoc test. * represents a p value < 0.05. ** represents a p value < 0.01.
Figure 3.3.14: Effect of corticosteroid treatment on extracellular Hsp72 levels in CLL patients.

Data is represented as normalised extracellular Hsp72 in serum from four representative CLL patients undergoing treatment with corticosteroids. Statistical Analysis was performed using the one-way ANOVA with Bonferroni’s post-hoc test. ** represents a p value < 0.01.
3.3.4 Heat Shock Protein 90 and Heat Shock Protein 27 Localisation in Chronic Lymphocytic Leukaemia

Expression of sHsp90 and sHsp27 was analysed on the leukocytes from CLL patients and control subjects. At no stage was Hsp90 or Hsp27 detectable on the surface of any cells (data not shown).

In both CLL and control samples, neutrophil, monocyte and lymphocyte populations were distinguished using FSC/SSC. A significant increase in total lymphocyte (CD5+/CD19+ and CD5-/CD19+ cells) iHsp90 expression was seen in CLL patients when compared to control subjects (P=0.0064) (Figure 3.3.15). Furthermore, this iHsp90 expression was shown to be dependent upon stage of disease as patients in Binet stage A showed significantly higher levels of Hsp90 expression than patients in Binet stages B or C (Figure 3.3.16). A significant increase in total lymphocyte iHsp27 expression was seen in CLL patients when compared to control subjects (P=0.0023) (Figure 3.3.17). However, in contrast to the trend observed for iHsp90, expression of iHsp27 was found to be independent of stage of disease as patients in Binet stage A, B and C showed similar levels of expression (Figure 3.3.18). Correlation analysis between both iHsp90 and iHsp27 and the level of active caspase-3 in the CLL cells revealed significant negative correlation between iHsp27 and caspase-3, but not Hsp90 and caspase-3 (P=0.0031, r=-0.5784) (Figure 3.3.19); Patients expressing higher levels of iHsp27 were found to display lower levels of active caspase-3.
Chapter 3: Heat Shock Protein Localisation in Chronic Lymphocytic Leukaemia

Figure 3.3.15: Intracellular Hsp90 expression in Lymphocytes from CLL patients and control subjects.
Hsp90 expression in total lymphocytes (CD5+/CD19+ and CD5-/CD19+ cells) from CLL patients and lymphocytes from control subjects was analysed by flow cytometry. Each group of patients is represented as a Mean of the MFI values, +/- SEM. Statistical Analysis was performed using the un-paired T-test.

Figure 3.3.16: Intracellular Hsp90 expression in Lymphocytes from CLL patients grouped according to Binet stage.
Intracellular Hsp90 expression in total lymphocytes (CD5+/CD19+ and CD5-/CD19+ cells) from CLL patients and lymphocytes from control subjects was analysed by flow cytometry. Each group of patients is represented as a Mean of the MFI values, +/- SEM. Statistical analysis was performed using the one-way ANOVA with Bonferroni’s post hoc test. * represents a p value < 0.05. ** represents a p value < 0.01. *** represents a p value < 0.001.
Figure 3.3.17: Intracellular Hsp27 expression in Lymphocytes from CLL patients and control subjects.

Hsp27 expression on total lymphocytes (CD5+/CD19+ and CD5-/CD19+ cells) from CLL patients and lymphocytes from control subjects. Each group of patients is represented as a Mean of the MFI values, +/- SEM. Statistical analysis was performed using the un-paired T-test.

Figure 3.3.18: Intracellular Hsp27 expression in Lymphocytes from CLL patients grouped according to Binet stage.

Intracellular Hsp27 expression in total lymphocytes (CD5+/CD19+ and CD5-/CD19+ cells) from CLL patients and in lymphocytes from control subjects was analysed by flow cytometry. Each group of patients is represented as a Mean of the MFI values, +/- SEM. Statistical analysis was performed using the one-way ANOVA with Bonferroni’s post hoc test. * represents a p value < 0.05.
Figure 3.3.19: Correlation analysis between intracellular Hsp27 and levels of active caspase-3 in total lymphocytes. CD5+/CD19+ and CD5-/CD19+ cells were gated. Correlation analysis was performed using Spearman’s correlation coefficient. $p = 0.0031$, $r = -0.5784$. 
3.3.5 ZAP-70 Expression in Chronic Lymphocytic Leukaemia

The presence of ZAP-70 in CD5+/CD19+ cells has previously been established as a marker of poor prognosis. The presence of ZAP-70 was analysed in the CD5+/CD19+ cells from these CLL patients.

Leukocytes from CLL patients were surface stained with CD5-APC and CD19-PE before intracellular staining with ZAP-70-PECy5. Malignant cells were gated and analysed for expression of ZAP-70 (Figure 3.3.20).

As ZAP-70 is a known client protein of Hsp90, it was hypothesised that there may be an association between the levels of iHsp90 displayed by CLL patients and the percentage of CLL cells expressing ZAP-70. A cut-off value of 20% has been widely accepted as the value to discriminate between a CLL sample that is ZAP-70 positive and a sample that is ZAP-70 negative (Crespo et al. 2003). Therefore, patients were grouped according to the number of CD5+/CD19+ cells in the sample expressing ZAP-70 and analysed for the expression of iHsp90. No significant difference was found between the two groups (Figure 3.3.21).
Figure 3.3.20: Expression of ZAP-70 in CD5+/CD19+ cells.
(A) Gating of malignant cells using CD5+ and CD19+ double staining in CLL sample. (B) and (C) CLL sample showing positivity for ZAP-70 in CD5+/CD19+ cells. Data is representative of 20 CLL patients.
**Figure 3.3.21: Intracellular Hsp90 in ZAP-70+ and ZAP-70- CLL patients.**
iHsp90 in total lymphocytes of CLL patients grouped according to the percentage of CD5+/CD19+ cells positive for ZAP-70. Data is represented as mean ± SEM. Statistical analysis was performed using the unpaired T-test.
3.3.6 T-Regulatory Cells in Chronic Lymphocytic Leukaemia

Numbers of T regulatory cells were analysed in PBMCs from CLL patients and control subjects. Cells were labelled with antibodies to CD4 and CD25 to detect a population containing activated T-cells and T-regulatory cells, then further labelled with an antibody to FoxP3 to detect the number of T-regulatory cells within the mixed population. CLL patients were found to possess a significantly higher number of circulating T-regulatory cells than control subjects (figure 3.3.22).

![Figure 3.3.22: Numbers of T-regulatory cells in PBMCs from CLL and control subjects.](image)

**Figure 3.3.22:** Numbers of T-regulatory cells in PBMCs from CLL and control subjects. Numbers represent the percentage of CD4+/CD25+ cells positive for FoxP3. Data is represented as mean ± SEM. Statistical analysis was performed using the unpaired T-test.
3.4 Discussion

The aim of this chapter was to explore both the internal and surface localisation of Hsp72, Hsp90 and Hsp27 in CLL patients and age-matched control subjects to determine if levels of these stress proteins had any clinical or prognostic significance.

3.4.1 Active Capsase-3 Levels in CLL

The levels of activated caspase-3, a marker of apoptosis, in total lymphocytes from CLL patients, was compared to levels expressed by control subjects, and was found to be significantly lower in CLL patients (Figure 3.3.1). This difference in apoptosis between CLL patients and control subjects is to be expected as the underlying basis of CLL is an inability for the B-lymphocytes to commit to apoptosis. There was, however, no difference in active caspase-3 between patients in different stages of the disease. This indicates that the progression of CLL is more likely to be the result of increased cellular clonal replication rather than an increased resistance to apoptosis.

3.4.2 Surface Heat Shock Protein Detection

Analysis of sHsp72 was performed using two different antibodies; a mouse monoclonal anti-human Hsp72 antibody (Stressgen SPA-810) and a mouse monoclonal anti-human TKD cmHsp70.1 antibody (Multimmune Ltd). The cmHsp70.1 antibody was raised against the TKD 14-mer, aa 450–463 peptide sequence (TKDNNLLGRFELSG) (Gehrman et al. 2003). This peptide has been shown to be exposed when the protein is embedded in the cell membrane. The epitope recognised by the Stressgen antibody remains hidden in the cell membrane when the protein is embedded. Washing leukocytes with a sodium chloride solution resulted in decreased antibody recognition when analysing with the Stressgen antibody (Table 3.3.4). This indicates that sHsp72 detected by the Stressgen antibody is loosely attached to the cell membrane, perhaps through protein receptors such as SR-A (Theriault et al. 2005).
Although Hsp72 and Hsp90 in particular have been observed on the surface of several tumour cell types (Multhoff et al. 1995; Gehrmann et al. 2003; Eustace et al. 2004), current opinion is that surface HSP expression is restricted to tumour cells (Multhoff et al. 1995; Multhoff & Hightower 1996; Multhoff et al. 2001). However, in the process of studying Hsp72 expression on leukocytes from CLL patients, the presence of Hsp72 was observed on the surface of normal, non-malignant cells. sHsp72 expression was demonstrated to be higher in the neutrophil population than the monocyte or lymphocyte populations. The data is consistent in that sHsp72 was seen on normal cells in both CLL patients and control subjects. The presence of sHsp72 on normal cells may relate to clearance of apoptotic cells by macrophages, as Hsp72 has been previously shown to interact directly with PS (Arispe et al. 2002; Vega et al. 2008). However, although PS is normally indicative of apoptosis, its presence has been shown on activated neutrophils, independent of apoptosis (Karmakar et al. 2005; Stowell et al. 2007). Therefore it could also be speculated that Hsp72 associated with PS may be masking PS from macrophages, thereby preventing binding to the macrophage and subsequent macrophage activation. Alternatively if sHsp72 is not associated with PS, it may be involved in neutrophil stability or may bind to scavenger receptors on macrophages thus initiating activation. Further data is necessary to analyse the function of sHsp72 on non-malignant cells, and the functional relationship between Hsp72 and PS.

In contrast to Hsp72, neither Hsp27 nor Hsp90 were detected on the surface of CD5+/CD19+ cells or non-malignant cells. This data conflicts with that of Becker et al., (2004), Eustace et al. (2004) and Sidera et al. (2008) who have demonstrated expression of sHsp90 on melanoma, fibrosarcoma and breast cancer cells respectively. However to date, the presence of Hsp90 or Hsp27 on the surface of normal cells has never been published.

3.4.3 Surface and Intracellular Heat Shock Protein Expression in CLL

On first consideration, the data presented here is consistent with the hypothesis that elevated intracellular HSP leads to tumour cells being resistant to apoptosis (Vargas-Roig et al. 1998; Nylandsted et al. 2000; Khaleque et al. 2005; Thomas et al. 2005). Expression of both intracellular Hsp90 and Hsp27 was found to be higher in CLL
patients (Figures 3.3.15 and 3.3.17), whilst activated caspase-3 was significantly lower when compared to control subjects. However, this analysis of the data maybe over-simplistic as it ignores the presence of sHsp72. The up-regulation of internal Hsp90 observed here in CLL patients is consistent with many other studies that have shown over-expression of this protein in numerous other cancer types and cancer cell lines (McCarthy et al. 2008; Yufu et al. 1992).

Hsp90 has been shown to stabilise many mutated kinases in tumour cells such as Bcr/Abl (An et al. 2000), FLT3 (Minami et al. 2002) and ErbB2 (Pashtan et al. 2008). Specific inhibition of Hsp90 in cancer cells causes rapid dissociation of these kinases from Hsp90 and consequent loss of kinase activity (Minami et al. 2002; Pashtan et al., 2008). Furthermore, although cancer cells may develop resistance to kinase inhibitors such as Imatinib due to mutations in the kinase binding domain, mutated kinases are still highly dependent upon Hsp90 for stabilisation and therefore still remain sensitive to Hsp90 inhibition (Gorre et al. 2002). Neckers, (2007), proposes that high levels of Hsp90 may provide cancer cells with the ability to survive in the presence of an unusually high mutation rate. The up-regulation of internal Hsp27 observed here agrees with work carried out on other cancers of the breast, liver and prostate that has also shown increased levels of this protein compared to control levels (Vargas-Roig et al. 1997; Cornford et al. 2000; Romani et al. 2007).

Hsp72, both surface and intracellular, was found to be expressed either at very high or very low levels in CD5+/CD19+ cells (Figure 3.3.6). The iHsp72 data showed a 1000-fold difference in expression between the two patient groups, although the difference in expression of sHsp72 was less pronounced. This variation between patients was demonstrated both by flow cytometry and western blot (Figures 3.3.6 and 3.3.9). Despite this huge variation in expression, there was no difference in Hsp72 levels between the different stages of the disease (Figure 3.3.11). Nevertheless, patients expressing high levels of sHsp72 or iHsp72 were found to be significantly different not only from patients expressing low levels of Hsp72, but also from levels expressed by non-malignant lymphocytes from CLL patients and lymphocytes from control subjects. However, neither sHsp72 nor iHsp72 could be correlated with stage of disease as patients in all three Binet stages appeared to express similar levels of iHsp72.
Further analysis of iHsp72 in CLL patients revealed that patients with stable disease (not requiring treatment) were displaying significantly higher levels of iHsp72 than patients with progressive disease (requiring treatment). Presence of high levels of iHsp72 in these less severe patients could be interpreted as a requirement, at the first stage of CLL, for cancer cells to survive any attempt by the immune system to restore normality. As the disease progresses, the cells replicate uncontrollably and at this stage, high levels of iHsp72 are no longer essential for cancer cell survival. Therefore the decrease of Hsp72 with severity of disease could potentially be used as a non-conventional marker for CLL progression, complementary to Binet staging and possibly improve prognostic accuracy in the early phases of the disease.

When levels of extracellular Hsp72 were analysed in serum from CLL patients and control subjects, it became apparent that there was also considerable variation in the degree of Hsp72 release from CLL cells. Grouping CLL patients as a whole, and drawing comparisons with control subjects showed no significant difference in release of Hsp72. However, further examination of the results revealed that patients who were receiving corticosteroid treatment displayed significantly lower levels of Hsp72 in serum when compared to patients not receiving steroid treatment (Figure 3.3.13). Indeed, levels of Hsp72 in serum from some steroid-treated patients were only just detectable by the ELISA, suggesting that steroid treatment may be inhibiting virtually all Hsp72 secretion. Examination of extracellular Hsp72 in individual CLL patients over time revealed a dramatic decrease in released Hsp72 after initiation of steroid treatment. Hsp72 levels in serum appeared to remain low throughout the treatment period (Figure 3.3.14). This data is supported by the work of Davies (2004) who demonstrated inhibition of Hsp72 release from Jurkat cells by Dexamethasone.

Although Hsp27, Hsp72 and Hsp90 have all been identified as having anti-apoptotic properties in tumour cells (Vargas-Roig et al. 1998; Nylandsted et al. 2000; Khaleque et al. 2005; Thomas et al. 2005), the relative expression of each HSP in tumour cells has not yet been investigated. This is surprising as these proteins interact at different levels, aside from the fact that all are induced by cellular stress. Hsp90 is essential for the stabilisation, activation and consequent function of a vast number of client proteins including many oncoproteins required for tumour cell
survival (Kamal et al. 2003; Becker et al. 2004). As a result several Hsp90 inhibitors such as GA and 17-AAG have been used to induce apoptosis in CLL cells with some success (Lin et al. 2007b; Pashtan et al. 2008). However, many of these compounds are toxic to cells and also result in activation of HSF1 which is generally bound to Hsp90. The result is an increase in Hsp27 and Hsp72 with consequent resistance to apoptosis (Ravagnan et al. 2001). Therefore it is important to establish if there is a concomitant Hsp72 and Hsp90 over-expression in order to inhibit both these proteins to obtain a tumour specific apoptosis. Recent work has highlighted the effectiveness of this strategy in a CML cell line where Hsp72 is over-expressed. The combination of the Hsp72 inhibitor resveratrol and 17-AAG increased the apoptosis of tumour cells (Chakraborty et al. 2008).

Resistance to corticosteroid therapy frequently displayed by CLL patients is thought to be attributed to imbalanced expression of glucocorticoid receptor (GR) isoforms. Indeed, higher expression of the transcriptionally inactive GR-β in relation to the hormone-activated transcription factor GR-α has been observed in CLL cells (Shahidi et al. 1999). However, another mechanism of steroid resistance may be defective ligand binding due to variations in the concentration of Hsp90 (Bailey et al. 2001). In a study on steroid resistance, it was shown that the ratio of Hsp90 to GR expression was significantly higher in steroid resistant, compared to steroid-sensitive multiple-sclerosis patients (Matysiak et al. 2008). This suggests that elevated levels of Hsp90 in the GR complex result in a reduced sensitivity to steroid therapy and points towards the possibility that patients in Binet stage A CLL, who express high levels of Hsp90, may display an increased resistance to steroids. With regards to Hsp72, release of this protein appears to be inhibited by steroid treatment and would suggest an internal increase in Hsp72. However, the number of steroid-treated patients analysed for iHsp72 was very low and so accurate conclusions cannot be drawn. Treatment with corticosteroids results in a reduction in antigen-presenting B-cells and so this may also account for the reduction in serum-Hsp72.

Several studies have shown the tyrosine kinase ZAP-70 to be a client protein of Hsp90 (Castro et al. 2005). It could be hypothesised therefore, that there may be an association between the levels iHsp90 and the level of ZAP-70 expression. However, grouping CLL patients according to ZAP-70 expression and analysing the level of
iHsp90 found no significant difference between the two groups. However, only 4 out of 20 patients could be considered negative for ZAP-70 and therefore accurate conclusions could not be determined. The unusually high number of patients positive for ZAP-70 may be due to the inclusion of a large number of advanced stage CLL patients who require regular monitoring and frequent treatment rather than early stage patients who attend the clinic less frequently. Furthermore, due to constraints on the use of specific fluorochromes, although ZAP70 was analysed in CD5+/CD19+ cells, iHsp90 was analysed in total lymphocytes (CD5+/CD19+ and CD5-/CD19+ cells) and therefore associations between the two proteins could not be accurately established.

Comparisons between the numbers of circulating Tregs in CLL patients and control subjects showed promising results. CLL patients were found to possess significantly higher numbers of Tregs than control subjects. This supports the work of Beyer et al. (2005), who showed not only elevated levels of Tregs in CLL patients but also found a relationship between numbers of Tregs and stage of disease. Although numerous studies have reported increased numbers of Tregs in solid tumours such as ovarian (Curiel et al. 2004) and breast cancer (Liyanage et al. 2002) it is surprising to find elevated levels of these cells in the peripheral blood of leukaemic patients due to the absence of a definite tumour site. However, it is unclear as to whether this increase in Treg numbers is a cause or effect; Does the observation of high numbers of Tregs in later stage disease indicate a commandeering of these naturally occurring immunosuppressors by the immune system, or, does the presence of Tregs at an early stage of disease allow the tumour to evade immune responses and progress further?

In summary Hsp72 was shown to be localised both inside and on the surface of leukocytes from CLL patients and control subjects and from patients in complete remission. Expression of Hsp90 and Hsp27 was shown to be restricted to the inside of the leukocytes from these patients. Although Hsp72 expression was observed in all patients, CLL patients could be divided into two significantly different groups based upon their expression of Hsp72; those expressing high sHsp72, and those expressing low sHsp72 on CD5+/CD19+ cells. Two groups of CLL patients were also observed when analysing iHsp72 expression in these malignant cells and was found to be related to whether the disease was stable or progressive. Analysis of Hsp72 in the serum of CLL patients revealed a relationship between low levels of
Hsp72 release and treatment with corticosteroids. Furthermore, although Hsp90 and Hsp27 could not be detected on the surface of leukocytes from CLL patients or control subjects, CLL patients showed significantly higher levels of internal Hsp90 and Hsp27 than those displayed by control subjects. iHsp90 expression was found to be associated with stage of disease, while iHsp27 was found to negatively correlate with active caspase-3.

There are two important consequences of the data presented here. Firstly, the significance of sHsp72 on normal, non-tumour cells needs to be explained. Secondly, considering that Hsp90 but not Hsp72 or Hsp27, is correlated with disease stage and that iHsp27 but not iHsp90 nor Hsp72 correlates with the level of active caspase-3, it is clear that these proteins have a dynamic relationship during development of CLL and the consequences for prognosis and treatment require further investigation.
3.5 Summary

- Caspase-3 levels were significantly lower in CLL patients when compared to control subjects.

- Treg cell numbers were significantly higher in CLL patients than control subjects

- CLL patients could be divided into two groups based upon their expression of sHsp72 and iHsp72; Very high levels or very low levels.

- Expression of iHsp72 was found to be associated with whether the disease was stable (not requiring treatment) or progressive (requiring treatment).

- CLL patients receiving corticosteroid treatment were found to release significantly lower levels of Hsp72 into serum

- Hsp27 levels were significantly higher in CLL patients than control subjects, and were found to negatively correlate with caspase-3.

- Hsp90 levels were significantly higher in CLL patients than control subjects, with patients in Binet stage A showing the highest levels of this protein.

A comparison between the levels of the analysed proteins and cells between CLL patients and control subjects can be seen in table 3.3.5

Table 3.3.5: Comparison between the levels of HSPs, caspase-3 and Tregs in CLL patients and control subjects. Data is presented as mean ± SEM.

<table>
<thead>
<tr>
<th></th>
<th>CLL Patients</th>
<th>Control Subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caspase-3</td>
<td>104.67 ± 16.51</td>
<td>247.12 ± 47.06</td>
</tr>
<tr>
<td>Tregs</td>
<td>42.64 ± 4.46</td>
<td>3.94 ± 0.645</td>
</tr>
<tr>
<td>Hsp27</td>
<td>2190.17 ± 188.00</td>
<td>1031.11 ± 219.13</td>
</tr>
<tr>
<td>Hsp90</td>
<td>2421.38 ± 307.76</td>
<td>722.75 ± 289.09</td>
</tr>
<tr>
<td>sHsp72</td>
<td>High: 11329.25 ± 2995.91 Low: 138.60 ± 34.71</td>
<td>646.28 ± 424.25</td>
</tr>
<tr>
<td>iHsp72</td>
<td>High: 1042.23 ± 253.64 Low: 0.001 ± 6.34x10⁻²⁰</td>
<td>254.44 ± 63.81</td>
</tr>
</tbody>
</table>