# Differential Leukocyte Heat Shock Protein Expression is Modulated in Health and Disease

By

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This Thesis is dedicated to Warren, Stephanie, Philip, Elliott and Callum.

#### **Declaration**

I certify that the substance of this thesis has not already been submitted for any degree and is not currently being submitted for any other degree or qualification.

I certify that to the best of my knowledge any help received in preparing this thesis, and all sources used, have been acknowledged in this thesis.



Linda Agnew

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#### Abstract

Heat shock proteins (hsps) are a group of highly conserved proteins that represent between 2% and 15% of total cellular protein and are expressed by every living organism. The main functions of hsps are to regulate apoptosis and to act as intracellular molecular chaperones that facilitate protein folding and assembly. Some hsps are highly immunogenic and elicit humoral, cytotoxic T-lymphocyte (CTL) and natural killer (NK) cell responses against viruses, tumors and infectious diseases.

In the first study, twenty male patients with HIV disease and fifteen age-matched controls were recruited. Lymphocytes were isolated and incubated at either  $37^{\circ}C$  for 1 h or heat shocked at  $42.5^{\circ}C$  for 1 h. Lymphocytes were then allowed to recover at  $37^{\circ}C$  for 3 h and hsp expression was measured using both western immunoblots and 1D-SDS-PAGE ( $\beta$ -actin used as internal control). After a mild heat shock (from  $37^{\circ}C$  to  $42.5^{\circ}C$  for 1 hr) lymphocytes displayed an augmented synthesis of hsp 110, hsp 90 and hsp 70, relative to actin, in all individuals regardless of HIV-status. There were apparent differences in levels of expression of newly synthesized hsps between the HIV-positive and HIV-negative groups. Within this cohort these differences were not correlated with CD4<sup>+</sup> count, viral load, dietary supplement use, smoking or the use of highly active antiretroviral therapy (HAART). The significance of altered hsp expression remains to be determined. However, given the recent reports on the role of these proteins in cross-presentation of antigens,  $\alpha$ -defensin internalization and pro-inflammatory cytokine production, further investigation is merited.

Flow cytometric methods have demonstrated that hsp70 is constitutively expressed in human leukocytes but that the level of expression varies considerably between different cell types. Specifically, monocytes express significantly more hsp70 than any other leukocyte subtype. These findings were confirmed and extended to develop a scientifically robust system for measuring hsp70 expression in leukocyte sub-populations that only required small volumes (1 - 2 ml) of whole blood. This methodology was then employed to compare hsp70 expression patterns in HIVpositive and HIV-negative cohorts. The results of this study demonstrated significant differences (p<0.05) in hsp70 expression at both basal and heat shock levels, in a number of leukocyte sub-populations, between HIV-positive (n = 42) and HIVnegative (n = 11) individuals. These differences were apparent in cytotoxic T cells (CD8+), helper T cells (CD4+) and monocytes (CD14+). There were no apparent differences in natural killer cell (CD56+) expression of hsp70. A common predictive factor for altered hsp70 expression was viral load suggesting that hsp70 expression may be a reflection of host-virus interactions resulting in alterations to the cellular stress response. Furthermore, preliminary findings indicated that leukocyte hsp70 expression may differ with disease progression/status as indicated by the patterns of hsp70 expression observed in the **HIV-positive** sub-groups examined (seroconverters, long-term non-progressors and Hepatitis C co-infected individuals). Further studies will be required before a definitive statement can be made, however, if the current findings are confirmed, leukocyte hsp70 expression may be a useful clinical marker for HIV-disease progression.

The immunomodulatory effects of oral dosing with an Echinacea preparation were investigated in a small scale clinical trial (n = 11). Expression of leukocyte heat shock protein 70 (hsp70), serum chemistry, haematological values and plasma alkylamide

concentrations were evaluated in eleven healthy individuals (26 to 61 years of age) at baseline (day 1) and on day 15 after consuming two commercially blended Echinacea tablets (containing both Echinacea angustifolia and Echinacea purpurea root) per day for fourteen days. Plasma alkylamide levels were determined one hour after ingestion of one Echinacea tablet and concentrations were found to be 12 ± 2 ng equiv/ml plasma. Echinacea supplementation significantly enhanced the fold increase from 2.2  $\pm$  0.4 to 3.3  $\pm$  0.7 (p = 0.03) in leukocyte hsp70 expression after a mild heat shock. Serum chemistry and haematological values for subjects after Echinacea supplementation did not vary significantly from baseline levels with the exception of white cell counts which increased from 6.6  $\pm$  0.4 to 7.2  $\pm$  0.3 x 10<sup>9</sup> (p = 0.04). Differential white cell counts displayed modest increases after Echinacea supplementation although only the lymphocyte sub-population approached significance (p = 0.06). The enhanced hsp70 stress response found was indicative of an improved immune response given that increases in hsp70 expression following cellular stress may play a critical role in antigen presentation, lymphocyte effector function and cytokine induction. This pilot study therefore suggested that supplementation with Echinacea may invoke an immune response through altered expression of hsp70 and increased white cell counts.

The effects of gender, menstrual cycle phase and oral contraceptive use on leukocyte hsp70 expression were examined in a small-scale study involving 11 female and 5 male participants. The results of these studies demonstrated that female leukocyte hsp70 expression displayed gender differences and in females, menstrual cycle phase differences and alterations as a result of oral contraceptive use were observed. These studies also demonstrated that serum leptin levels displayed gender dimorphism, serum cortisol levels were higher in females using oral contraceptives, and that serum prolactin levels displayed menstrual cycle variation in females and showed gender dimorphism. This is the first report of changes in leukocyte hsp70 expression in response to gender, menstrual cycle phase and oral contraceptive use indicating interactions between the immune response and stress response pathways.

#### **Publications arising from this Thesis**

Agnew LL, Kelly M, Howard J, Jeganathan S, Batterham M, Ffrench RA, Gold J, Watson K. Altered lymphocyte heat shock protein 70 expression in patients with HIV disease. *AIDS*, 2003; **17(13):** 1985-1988. A copy of this paper appears in Appendix III.

Agnew LL, Guffogg SP, Matthias AM, Lehmann RP, Bone K, Watson K. Echinacea intake induces an immune response through altered expression of leukocyte hsp70, increased white cell counts and improved erythrocyte antioxidant defences. *Journal of Clinical Pharmacy and Therapeutics* 2005; **30:** 363-369. A copy of this paper appears in Appendix III.

Agnew LL. And Watson K. Stress Proteins as Biomarkers of Oxidative Stress. *Current Protocols in Toxicology* 2006; Supplement 28, **17.8**: 1 – 25. A copy of this paper appears in Appendix III.

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#### Aspects of Thesis presented at conference proceedings

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Poster "HIV-positive individuals have altered hsp70 expression in a number of leukocyte subtypes" at the Gordon Research Conference Stress Proteins in Health, Development and Disease, Newport, Rhode Island, USA, 2005. Also presented at the School of Biological, Biomedical & Molecular Sciences, Postgraduate Conference, University of New England, Armidale Australia, 2006

Poster "Effect of Echinacea on human immune responses *in vivo*" Presented at the 53<sup>rd</sup> Annual Congress of the Society for Medicinal Plant Research, Florence, Italy, 2005.

Abstracts and copies of these posters are presented in Appendix II.

#### Abbreviations

- **AAPH** 2,2'-azobis-(2-amidinopropane) dihydrochloride
- ACTH adrenocorticotropic hormone
- ADCC antibody-dependent cellular cytotoxicity
- AIDS acquired immune deficiency syndrome
- AMPS ammonium peroxodisulphate
- APC Allophycocyanin
- **APC** antigen presenting cell
- APC-Cy7 Allophycocyanin-Cy7
- ASC Albion Street Centre
- BSA bovine serum albumin
- CAM complementary and alternative medicine
- CHD cardiovascular heart disease
- CFI Centre for Immunology, St Vincent's Hospital, Sydney
- CNS central nervous system
- **CO** carbon monoxide
- CTL cytotoxic T lymphocyte
- **DC** dendritic cell
- DMSO dimethyl sulfoxide
- DNA deoxyribo nucleic acid
- **DNPH** 2,4-dinitrophenylhydrazine
- ELISA enzyme linked immunosorbent assay
- **ER** endoplasmic reticulum
- FACS flow cytometry and cell sorting
- FCS fetal calf serum
- FITC Fluorescein
- **FSH** follicle stimulating hormone
- **GnRH** gonadotrophin-releasing hormone
- grp glucose regulated protein
- **GSH** glutathione
- **HAART** highly active antiretroviral therapy
- HIV human immunodeficiency virus
- HLA human leukocyte antigen
- HO-1 heme oxygnase

| HPA    | hypothalamic-pituitary-adrenal axis            |
|--------|--|
| HRT    | hormone replacement therapy                    |
| HSE    | heat shock element                             |
| HSF    | heat shock factor                              |
| hsp    | heat shock protein                             |
| HTLV   | human T-lymphocytotropic virus                 |
| IFN-γ  | interferon-gamma                               |
| IL     | interleukin                                    |
| kDa    | kilodalton                                     |
| LAV    | lymphadenopathy associated virus               |
| LH     | luteinizing hormone                            |
| LPS    | lipopolysaccharide                             |
| LTNP   | long-term non-progressor                       |
| МАРК   | mitogen-activated protein kinases              |
| MDA    | maliondialdehyde                               |
| МНС    | major histocompatibility complex               |
| MS     | multiple sclerosis                             |
| MUFA   | monounsaturated fatty acids                    |
| NAC    | N-acetylcysteine                               |
| NK     | natural killer cells                           |
| NF-κB  | nuclear factor- κΒ                             |
| NNRTI  | non-nucleoside reverse transcriptase inhibitor |
| NO     | nitric oxide                                   |
| NRTI   | nucleoside reverse transcriptase inhibitor     |
| oc     | oral contraceptive                             |
| PBMC   | peripheral blood mononuclear cells             |
| PBS    | phosphate buffered saline                      |
| PBS-T  | phosphate buffered saline-Tween 20             |
| PE     | Phycoerythrin                                  |
| PE-Cy7 | Phycoerythrin-Cy7                              |
| PerCP  | Peridinin Chlorophyll Protein                  |
| PI     | protease inhibitor                             |
| PLWHA  | people living with HIV/AIDS                    |
| PMSF   | phenylmethylsulphonyl fluoride                 |

| PUFA     | polyunsaturated fatty acid                                  |
|----------|---|
| RNA      | ribonucleic acid  |
| ROS      | reactive oxygen species                                     |
| SD       | standard deviation  |
| SDS      | sodium dodecyl sulphate                                     |
| SDS-PAGE | sodium dodecyl sulphate-poly acrylamide gel electrophoresis |
| SEM      | standard error of the mean                                  |
| SFA      | saturated fatty acid  |
| SIV      | simian immunodeficiency virus                               |
| SLE      | systemic lupus erythematosus                                |
| STD      | sexually transmissible disease                              |
| STI      | structured treatment interruption                           |
| TAS      | total antioxidant status                                    |
| TBARS    | thiobarbituric acid reactive substances                     |
| ТСА      | trichloroacetic acid  |
| TCR      | T cell receptor   |
| TEMED    | N,N,N',N',-tetramethylenediamine                            |
| TLR      | toll-like receptor  |
| TNF-α    | tumour necrosis factor alpha                                |
| WRL      | Westfield Research Laboratories                             |

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