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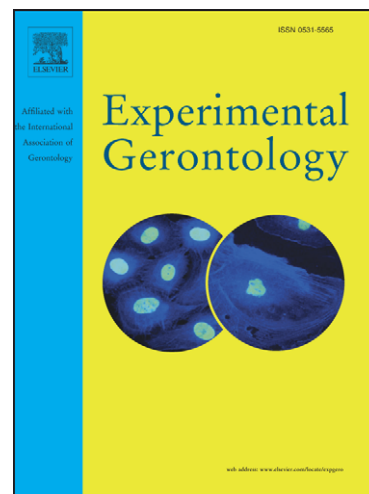
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Abstract

Chaperones and zinc are indispensable for proper immune function. All the zinc status, the immune function and the stress response decline during aging. Here we studied the effect of nutritional zinc and zinc homeostasis on the stress response in healthy old subjects recruited during the ZincAge European Union project that either underwent or not a 48-day zinc supplementation. Inducible Hsp70 levels were determined at basal conditions as well as after heat shock in the CD3+ and CD3- subset of lymphocytes by a two-color FACS analysis. Short term zinc supplementation resulted in a marked increase in both basal as well as stress-induced Hsp70 levels in lymphocytes from healthy elderly donors with a higher impact on CD3+ cells. Heat inducibility showed a strong correlation with basal Hsp70 level, and both basal as well as stress-induced Hsp70 highly correlated with intracellular zinc availability. In conclusion, short-term oral supplementation with zinc safely and efficiently induces the stress response in lymphocytes of old donors. The stress response may be a candidate pathway connecting zinc deficiency with aging and immunosenescence. Thus, proper dietary zinc intake may emerge as a chaperone inducer and an anti-aging mechanism in the immune system.

1. Introduction

Zinc is one of the most important trace elements in the body (Stefanidou et al., 2006). It has a catalytic/regulatory role in many enzymes, maintains the structural integrity of various proteins (such as superoxide dismutase and zinc-finger transcription factors) and modulates protein-protein interactions. At the cellular level zinc is essential for cell proliferation and survival, contributes to genomic stability and antioxidant defense, which highlights its crucial role in aging and age-dependent degenerative diseases. Zinc is indispensable for proper immune function. Zinc deficiency increases susceptibility to infections, compromises both innate immunity and the T-cell compartment (Shankar and Prasad, 1998; Rink and Haase, 2007). It is well documented that zinc status and immune function declines with aging, and zinc supplementation is beneficial to immune responses in the elderly (Haase et al., 2006b; Larbi et al., 2006).

Molecular chaperones are conserved and abundant proteins that guard the conformational homeostasis of proteins (Hartl, 1996). They maintain signaling, regulate proliferation, differentiation and apoptotic pathways (Sóti et al., 2005b; Sreedhar and Csermely, 2004). Chaperones (or stress proteins) confer cytoprotection and assure survival upon various stresses. The stress response is regulated by the heat shock transcription factor 1 (HSF-1)¹, named after the archetype of proteotoxic stress. HSF-1 induces the transcription of various stress proteins. Inducibility of the major chaperone, the 70 kDa heat shock protein (Hsp70) reflects the robustness of the stress response, which is indispensable for adaptation (Voellmy, 2004). Both a single heat shock as well as transgenic Hsp70 induces longevity (Tatar et al., 1997), HSF-1 overexpression induces a twofold life-span extension, while HSF-1 knock-out markedly shortens life-span (Hsu et al., 2003; Garigan et al, 2002), demonstrating the central importance of a robust stress response in aging in invertebrate models. HSF-1 knock-out mice neither display heat shock response, nor show premature aging in ‘sterile’

1 laboratory conditions. However, they are hypersensitive to endotoxemia, which reinforces the
2 role of the stress response in immune function and inflammation (Xiao et al., 1999).

3 Moreover, both chaperone inducibility and chaperone function decrease during aging and in
4 chronic inflammation characteristic to the elderly (Nardai et al., 2002; Söti and Csermely,
5 2003; Arslan et al., 2006), therefore preserving their function is an attractive target in anti-
6 ageing therapies (Söti et al., 2005a; Söti and Csermely, 2005).

7 Zinc and chaperones are connected in many ways (Larbi et al., 2006; Arslan et al.,
8 2006; Söti and Csermely, 2005). Zinc is a potent inducer of Hsp70 in cell culture (Hatayama
9 et al., 1993) including lymphoblasts from old, but not from young donors (Ambra et al.,
10 2004). Both zinc deficiency and overdose inhibits the stress response in rodent thymus (Moore
11 et al., 2003). Thus, proper dietary zinc is critical for mounting a robust stress response. The
12 ZincAge project funded by the 6th Framework Program of the European Union aimed to study
13 the role of nutritional zinc in healthy aging and in immunosenescence. We were interested to
14 study the relationship of zinc homeostasis and aging with the stress response, an essential
15 adaptive and survival mechanism in the immune system. Here we report on the effect of zinc
16 status on the stress response of lymphocytes obtained from healthy elderly donors.

2. Materials and methods

2.1. Subjects and zinc supplementation

Twenty healthy old subjects (between 64 and 85 years of age) were enrolled according to the inclusion criteria required by the ZincAge European specific targeted research project (www.zincage.org) approved by the respective National Ethical Committees. Briefly, all subjects were originated from Italy, lived an independent life, were in a good general health conditions devoid of functional impairment and serious acute or chronic disease, without taking medical drugs or nutritional supplements. All subjects gave informed consent and underwent medical examination and clinical laboratory tests. Seven persons from the twenty healthy old subjects underwent an oral zinc supplementation which was performed with 10 mg pure zinc/day (in the form of 50 mg zinc-aspartate Unizink 50, KÖHLER PHARMA Corp., Alsbach-Hähnlein, Germany) for 48 ± 2 days. All subjects admitted to zinc supplementation had values of plasma zinc below $11 \mu\text{M}$ at least at one test out of two made within one year.

2.2. Blood withdrawal and PBMC preparation

Heparinized whole blood samples after an overnight fast were withdrawn from each subject before and after zinc supplementation. The whole blood was centrifuged at $450 \times g$ for 10 min at 4°C to separate the plasma. Peripheral blood mononuclear cells (PBMC) were prepared by Ficoll-Hypaque ($d = 1.077 \text{ g/ml}$) gradient centrifugation ($450 \times g$ for 30 min at 20°C), washed twice in PBS, and aliquots were made in fetal bovine serum containing 10% DMSO and were frozen in liquid nitrogen until further use.

2.3. Plasma zinc and intracellular zinc ion availability determination

1 Plasma zinc levels were analyzed by induction plasma coupled mass spectrometry
2 (ICP-MS). All plasma samples and standard were diluted 1:10 with a solution containing the
3 following reagents: 0.1% Triton-X100, to maintain a stable emulsion with the diluted sample
4 and 0.15 % HNO₃, to ensure that trace elements are maintained in solution and to aid washout
5 of these elements between samples. External calibration solutions containing zinc (blank to
6 2000 ppb), were prepared by serial dilution of parent 1000 ppm stock (VHG Labs,
7 Manchester, NH, USA), using the same solution used to dilute the samples. Measurement of
8 plasma zinc was performed with a Thermo XII Series ICP-MS (Thermo Electron Corporation,
9 Waltham, MA, USA), using an external calibration curve containing zinc (blank to 2000 ppb).
10 The instrument was operated with a Peltier-cooled impact bead spray chamber, single piece
11 quartz torch (1.5 mm i.d. injector) together with Xi interface cones and a Cetac-ASX 100
12 autosampler (CETAC Technologies, Omaha, Nebraska, USA). A Burgener Trace nebuliser
13 was used as this device does not block during aspiration of clinical samples. The instrument
14 was operated in standard mode (non-CCT), using 1400 W RF power, 1.10 L/min nebuliser gas
15 flow, 0.70 L/min auxiliary gas flow, 13.0 L/min cool gas flow, 70 ms dwell time, 30 s sample
16 uptake and 35 s wash time (2 repeats per sample). Zinc ion availability of PBMC was
17 measured with zinpyr-1 (from Neurobiotex, Galvestone, TX, USA) by flow cytometry, as
18 described (Malavolta et al., 2006).). Briefly, PBMCs (1×10^6) were stained with 20 μ M
19 Zinpyr-1 (ZP1) (Neurobiotex, Galveston, Texas, USA) for 30 min at 37C and 5% CO₂ in
20 HEPES-buffered "zinc-free" RPMI Medium supplemented with 1 mM EDTA. Following
21 incubation, cells were analyzed with a flow cytometer (Coulter Epics XL, Coulter, USA). The
22 population of lymphocytes was selected using FSC and SSC and the ZP1 derived fluorescence
23 was detected at 525 nm using 488 nm excitation wavelength. Data for zinc ion availability are
24 reported as the mean fluorescence intensity normalized to the minimum fluorescence obtained

1 after the addition of 50 μ M N,N',N'-tetrakis (2-pyridylmethyl)ethylenediamine (TPEN)
2 (Sigma–Aldrich, MI, Italy).

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PBMC were gently thawed in RPMI 1640 medium, supplemented with 10 mM Hepes,
10% fetal bovine serum, 2 mM L-glutamine, 100 μ g/ml streptomycin and 100 IU/ml penicillin
(all from Gibco-Invitrogen, Carlsbad, CA, USA) and incubated for 16 hours at 37 °C in a 5%
CO₂ incubator at isobaric oxygen. Each PBMC sample was divided into control and heat
shock aliquot. Heat shock was performed at 43 °C for 1h in an Eppendorf thermomixer at a
cell density of 10⁶/ml. The induction of the heat shock response was allowed to develop for 4
h at 37 °C in a 5% CO₂ incubator. Control PBMCs of the same person were treated identically
except for exposing the cells to heat shock. Then cells were harvested, washed with PBS
supplemented with 2% fetal bovine serum, counted and the viability was assessed using a
CASY TT cell counter (Scharfe Systems, Bielefeld, Germany). Viability was over 90%.

17 **2.5. Intracellular staining and flow cytometry**

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Cells were fixed and permeabilized in 250 μ l Cytotfix/Cytoperm solution (BD
Biosciences Pharmingen, San Diego, CA, USA) at 4 °C for 20 minutes. Cells were then
washed two times with Perm/Wash solution (BD Biosciences Pharmingen, San Diego, CA,
USA) and incubated with flouochrome-conjugated mouse monoclonal antibodies [anti-CD3
allophycocyanin (APC) conjugated antibody (345767, BD Biosciences, San Diego, CA, USA)
and anti-Hsp70 fluorescein isothiocyanate (FITC) conjugated antibody (SPA-810FI,
Stressgen, Alberta, Canada)] at 4 °C for 30 minutes. (CD3 immunostaining either in intact
non-permeabilized or in permeabilized cells gave identical results.) For compensation cells

1 were stained either with anti-CD3-APC conjugated antibody or with anti-CD3 FITC
2 conjugated antibody (345763, BD Biosciences, San Diego, CA, USA) as described above.
3 After washing once with Perm/Wash solution and once with PBS with 2% FBS cells were
4 fixed with 2% paraformaldehyde in PBS and analyzed with a FACSCalibur flow cytometer
5 using the CellQuest software (BD Biosciences, San Diego, CA, USA). Approximately 5000
6 live lymphocytes from each sample were electronically gated according to granularity and size
7 in the forward versus side scatter (**Figure 1A**). Differentiation between CD3+ and CD3-
8 subpopulations was achieved in the CD3-APC vs. side scattergram (**Figure 1B**). Hsp70-
9 related fluorescence was obtained in a 530/30 (FITC) filter, and the relative Hsp70 protein
10 level was expressed as the mean fluorescence intensity (MFI) of FITC from logarithmic
11 histograms. As a standard, two parallel vials of lymphocytes isolated from the buffy coat of a
12 healthy subject (purchased from the Hungarian National Blood Transfusion Service, approved
13 by the National Ethical Committee, and in accordance with the Helsinki regulations) were
14 used in each experiment including heat shock treatment, staining and FACS analysis.

16 ***2.6. Statistical analysis***

17 Data were statistically analyzed using Statistical Package for the Social Sciences
18 software version 15.0 for Windows (SPSS Inc., Chicago, IL, USA). Variables are expressed as
19 mean \pm standard error of the mean (SEM). Means were either compared by using the
20 Student's paired samples or independent samples t-test. Correlation of continuous variables
21 was analyzed by the Spearman algorithm. A p value (two-tailed) < 0.05 was considered
22 statistically significant.

3. Results

3.1. Characteristics of the stress response in lymphocyte populations

In order to gain insight into the stress response in specific subpopulations of lymphocytes, we set up a two-channel FACS analysis, using CD3 (**Figure 1AB**), a T-lymphocyte marker, and Hsp70, the major cytosolic stress protein. Initial experiments were performed on pooled PBMC-s from healthy donors. While basal Hsp70 level reflects steady-state chaperone capacity, stress-induced Hsp70 level determines the robustness of adaptation to a noxious insult. Heat shock, a model of fever was selected not only as the archetype of stress but also as an important physiopathological determinant of lymphocyte function. The optimal conditions of induction were found to be 43°C for 1 hour and 4 hours of recovery time to allow Hsp70 synthesis (data not shown) and used in subsequent comparative studies.

Looking at the Hsp70 distributions, a high and a low Hsp70-expressing population could be observed, and the high population could respond more dynamically to heat shock (**Figure 1C all, 37 vs. 43°C**). Further examining the relationship between baseline and heat-induced Hsp70 levels, we investigated the stress response in PBMC obtained from old donors. We found a significant correlation between these variables in the CD3+ lymphocyte populations, while no such strong association existed in CD3- lymphocytes (**Figure 2**). Moreover, the strong correlation persisted with respect to inducibility, indicating that it is indeed a relationship between the basal level and the mounting of the stress response. We did not observe any gender-specific effect.

3.2. Zinc supplementation boosts the stress response

Next, we tested the effect of clinical zinc supplementation on the stress response. Seven healthy elderly subjects received 10 mg zinc/day for 48 ± 2 days, and blood was withdrawn before and after the supplementation. Zinc supplementation did not significantly

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4 1 increase plasma zinc concentration, however, it promoted a significant improvement of zinc
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6 2 availability, an intracellular measure of free zinc ion available for cellular reactions, therefore
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8 3 a better marker of zinc homeostasis (**Table 1**). **Figure 3** shows the individual characteristics
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10 4 of the stress response in the total lymphocyte population before and after zinc
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12 5 supplementation. All the basal (panel A) and heat-induced Hsp70 levels (B) as well as the
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14 6 inducibility (C) were boosted by oral zinc supplementation, as can be seen from the upward
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16 7 trend of the individual subjects. However, heat induction of Hsp70 showed a more uniform
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18 8 and stronger increase.

22 9 Statistical analysis showed a significant increase of the heat-induced Hsp70 as well as
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24 10 the stress-inducibility upon zinc supplementation, while the basal Hsp70 level displayed a
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26 11 tendency to increase, probably due to the limited number of subjects (**Figure 4**). Zinc affected
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28 12 the CD3- population to a smaller extent. It should be also noted that CD3- lymphocytes
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30 13 displayed a higher basal level but a less intense heat induction of Hsp70 than CD3+ cells
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32 14 (**Figure 4**). Furthermore, the lymphocyte fraction that highly express Hsp70 (gated at Hsp 70
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34 15 MFI>18) showed a sharp increase both under basal as well as heat-induced conditions upon
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36 16 zinc supplementation (basal: $9.6 \pm 2.9\%$ before zinc vs. $22.1 \pm 6.3\%$ after zinc, $p = 0.026$;
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38 17 heat-induced: $22.0 \pm 16.7\%$ before zinc vs. $57.8 \pm 5.5\%$ after zinc, $p = 0.004$, respectively).

44 18 To further investigate the effect of orally taken zinc on the stress response, we
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46 19 compared the Hsp70 status of subjects (Age: 76.6 ± 1.7 , $n = 10$) after zinc supplementation
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48 20 with that of subjects (Age: 72.2 ± 1.8 , $n = 10$, $p = 0.095$ for age) not having taken zinc tablets.
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50 21 **Figure 5** demonstrates that zinc-supplemented subjects had higher Hsp70 levels and
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52 22 inducibility in all lymphocyte populations.

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58 24 **3.3. Robustness of the stress response correlates with intracellular zinc**
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60 25 **availability**

1 After having seen the effect of clinical zinc supplementation, we were interested to
2 analyze the relationship between zinc status and Hsp70 levels/inducibility. We clustered the
3 subjects according to either plasma zinc or intracellular zinc availability in low or high
4 groups. Subjects with high zinc availability displayed a higher steady state Hsp70 level and a
5 more robust stress response. Plasma zinc levels did not significantly differ between groups
6 (**Table 2**). Using the same subjects, further calculation of bivariate linear correlations of basal
7 Hsp70 vs. zinc availability and heat-induced Hsp70 vs. zinc availability showed that both the
8 basal as well as the heat-induced Hsp70 level correlated significantly with intracellular zinc
9 availability in a concentration-dependent manner (basal Hsp70 vs. intracellular zinc
10 availability: $R^2=0.3260$, $p=0,005$; heat induced Hsp70 vs. intracellular zinc ion availability:
11 $R^2=0,4137$, $p=0,002$). In contrast, plasma zinc level seemed to have no significant correlation
12 with heat induced Hsp70 levels ($R^2=0,0048$, $p=0,772$). We observed no gender-specific
13 effect.

15 **4. Discussion**

16 Loss of adaptation to stress is a hallmark of aging. A major molecular mechanism
17 behind is a decrease in heat shock response, a fundamental cytoprotective and survival
18 mechanism in several models (Sóti and Csermely, 2003; Arslan et al., 2006), including human
19 blood cells and extracellular chaperones of the elderly (Njemini et al., 2002; Rea et al., 2001;
20 Singh et al., 2006). Since molecular chaperones are intimately involved in signaling and
21 proliferation, a less robust stress response may not only cause limited survival, but may also
22 contribute to the compromised reactivity of old lymphocytes to various inflammatory stimuli,
23 a phenomenon called immune cell anergy (Pawelec, 2006). Besides, looking at two
24 lymphocyte subpopulations it turned out that CD3⁻ cells had a significantly higher basal and a
25 lower heat-induced Hsp70 level than their CD3⁺ counterparts. Whether the CD3⁻ B-cells (and

1 NK-cells) have almost no stress tolerance (in terms of heat shock response) only in elderly
2 subjects and if it relates to immune function is an intriguing open question.

3 Stress tolerance, the adaptation to an environmental noxa depends on the robustness of
4 the stress response, i.e. the inducibility of stress proteins by the heat shock transcription factor
5 (Voellmy, 2004). Preconditioning (exposure to a mild stress, like hormesis [Verbeke et al.,
6 2001; Rattan, 2004]) results in elevations in Hsp70 and other stress proteins, a more robust
7 stress response and a better adaptation with implications in longevity. However, large
8 elevation in Hsp70 is only transient and the remaining protein level is insufficient to meet the
9 increased demand during stress. To our knowledge, no molecular mechanism has been
10 associated with a better mounting of the stress response after a previous/chronic mild stress.
11 We observed that basal Hsp70 level in the CD3+ lymphocytes strongly correlates ($R^2 =$
12 0.6102) with its own inducibility (**Figure 2A**). Our results imply that Hsp70 may indeed
13 operate a positive feedback loop chaperoning its own synthesis upon a subsequent stress,
14 providing a framework for stress tolerance. Furthermore, one can predict that cells possessing
15 higher basal Hsp70 levels might have been primed by hormetic means and have better chances
16 to adapt to stress. On the contrary, continuously very high level, such as strong overexpression
17 of Hsp70 would compromise cell physiology (Seo et al., 1996), but this threshold either may
18 not be reached in physiological setting or in the old cells analysed in the current study. Further
19 studies are needed to explore the molecular details and its applicability as a biomarker of
20 stress tolerance.

21 Zinc is indispensable for proper immune response. Zinc deficiency is detrimental,
22 while zinc supplementation is beneficial to T-lymphocyte function (Shankar and Prasad, 1998;
23 Rink and Haase, 2007). Both zinc status and immune function declines with aging (Haase et
24 al., 2006b; Larbi et al., 2006). Here we obtained evidence that nutritional zinc intake
25 modulates the stress response. Short term zinc supplementation markedly improved all aspects

1 of lymphocyte Hsp70 homeostasis involving also CD3- cells, in spite of their less reactivity to
2 heat shock (**Figures 4**). This effect was highly significant even in a relatively small study
3 population. The observations were extended and confirmed towards subjects that were or were
4 not supplemented with zinc (**Figure 5**). Intriguingly, plasma zinc was mostly in the normal
5 range for the subjects studied (**Tables 1 and 2**) and there was no significant difference of
6 Hsp70 response between subjects having lower and higher than 11 μM plasma zinc
7 concentration, respectively (data not shown). In contrast, intracellular zinc availability highly
8 correlated with basal and heat-induced Hsp70 levels, strongly suggesting that low zinc
9 availability exerts a negative effect on the stress response, and even without subclinical zinc
10 deficiency (plasma Zn < 11 μM) zinc supplemented subjects show a more robust Hsp70
11 induction. Notably, plasma zinc concentration did not seem to affect Hsp70 inducibility. This
12 may be in part attributed to the limited number of subjects and in part to the fact that plasma is
13 often discussed not to be reliable to monitor zinc status in humans, because zinc occurs in the
14 body primarily intracellularly and there is only a small portion of zinc in the plasma mainly
15 bound to plasma proteins. Furthermore, plasma zinc is highly dynamic and is profoundly
16 affected by several factors such as diurnal rhythm, stress, infection, starvation and plasma
17 protein levels (Wood, 2000). Alternatively, zinc status is measured by erythrocyte membrane
18 zinc (Bettger et al., 1986) or metallothioneins (Sullivan et al., 1998), proteins which are
19 responsible for the regulation of intracellular free zinc ions. Similarly to our results, Thomas
20 et al. (1992) showed that metallothionein concentrations in erythrocytes elevated significantly
21 while no changes in plasma zinc could be observed after zinc supplementation. Recent *in vitro*
22 assessments (Malavolta et al., 2006; Haase et al., 2006a) and preliminary “*in vivo*” findings
23 (Mocchegiani, 2007; Cipriano et al., 2006) suggest that intracellular zinc availability (or labile
24 zinc) of leukocytes may be a parameter that allows a good determination of zinc status. The
25 intracellular zinc that is available for zinc-dependent enzymes is often called as the labile pool

1 of zinc which is modulated by the release of zinc from intracellular vesicular stores or from
2 metallothioneins what is essential to regulate many cellular processes such as apoptosis, stress
3 response or cell cycle (Jacob et al., 1998; Malavolta et al., 2006). Moreover, since stress
4 signals mobilize zinc by oxidation of protein sulphur ligands (Maret, 2006), the assessment of
5 labile zinc represent an optimal target for establishing a link between stress response and zinc
6 status. In addition to these, this method allowed also the determination of zinc in the cells
7 analyzed while plasma zinc is not a cell specific indicator of zinc status. Our findings
8 corroborates *in vitro* experiments that reported a stress response inducing property of zinc in
9 various cell cultures and peripheral cells of different origin (Hatayama et al., 1993; Ambra et
10 al., 2004; Bauman et al., 1993; Odashima et al., 2002; Quing et al., 2004 and reviewed in
11 Arslan et al., 2006). Similarly, zinc in our study also increased the basal steady-state Hsp70
12 level in human lymphocytes, suggesting that zinc per se exerts a mild stress on a variety of
13 cell types. This property of zinc might resembles an hormetic action (Rattan, 2004). In fact,
14 the zinc-induced increase in Hsp70 elicited a more intense stress response (2-4-fold increase
15 of heat-induced Hsp70) with no apparent damage. Moreover, PBMC from zinc-supplemented
16 people had much better morphology (data not shown), arguing for a generally improved
17 ability to cope with stress. Since blood samples were obtained and frozen under controlled
18 conditions at different times by the same personnel, the effect observed was not due to
19 differential sample preparation.

20 Chaperones and zinc may co-operate in other ways, as well. Besides, zinc is also an
21 important regulatory metal in protein function and signaling. Both zinc and chaperones
22 promote lymphocyte proliferation (Csermely et al., 1988; Huse et al., 1998; Schnaider et al.,
23 2000). Lack of zinc results in decreased cell proliferation, skin and mucous membrane
24 manifestations and decreased wound healing (Prasad, 2003), while chaperone upregulation
25 facilitates wound healing and regeneration (Vigh et al., 1997). Moreover, zinc deprivation

1 compromises the stress response in keratinocytes, and in the Jurkat T-cell line (Larbi et al.,
2 2006; Parat et al., 1998). An upregulation of HSF-1 was observed in T-cell clones both upon
3 zinc deprivation and during *in vitro* aging, suggesting a compensatory mechanism. As HSF-1
4 is a zinc finger protein, it may be directly affected by fluctuations in intracellular free zinc ion
5 availability. This is supported by the fact that free intracellular zinc is in the femtomolar
6 range, around the K_D of various zinc-binding proteins (Outten et al, 2001) and p53 and the
7 apurinic/apyrimidinic endonuclease are all inactivated by zinc deficiency (Ho and Ames,
8 2002; Ho et al., 2003). According to a recent intriguing hypothesis of Bruce Ames (Ames,
9 2006), shortage of micronutrients may promote the re-allocation of scarce micronutrients
10 towards processes favoring short term survival at the expense of longevity. According to this,
11 the stress response (a major adaptation mechanism) would either enjoy an intermediate
12 priority (after basic cellular processes like oxidative metabolism, and before reproduction) and
13 as such, would be downregulated during modest nutrient deprivation, as seen in the present
14 study. It is very much in agreement with the fundamental role of the stress response in
15 longevity (Sóti and Csermely, 2003). Therefore, beyond genomic stability, the stress response
16 is another candidate mechanism to connect nutrient scarcity with aging and degenerative
17 diseases, at least in the cases of zinc. Effect of other micronutrients on the stress response
18 remains to be elucidated.

19 As a conclusion, we provided evidence that dietary zinc is a chaperone inducer and a
20 major determinant of the stress response in human lymphocytes in the elderly. Since the stress
21 response is a conserved and universal phenomenon, and the effect of zinc *in vitro* is fairly
22 general, these findings may have wider implications in other bodily tissues as a possible anti-
23 aging mechanism. However, whether this is a proof of principle or a special exception will be
24 a subject of future studies.

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¹Abbreviations used: HSF-1, heat shock transcription factor 1; Hsp70, 70 kDa heat shock or stress protein, MFI, mean fluorescence intensity, PBMC, peripheral blood mononuclear cells

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4 **Tables**
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	<i>Age (years)</i>	<i>Plasma Zn (μM)</i>	<i>Zn availability</i>
Before Zn suppl.	74.7 \pm 2.0	12.1 \pm 0.7	1.26 \pm 0.03
After Zn suppl.	-	13.5 \pm 1.5	1.39 \pm 0.02
P	-	0.367	0.013

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23 **Table 1 Age of the subjects and zinc status before and after zinc supplementation.**

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25 Data (n = 7) were obtained as described in Subjects and methods and shown as mean \pm SEM.

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27 p values were calculated using the Student's paired samples t-test.
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<i>Group (n)</i>	<i>Plasma Zn (μM)</i>	<i>Zn availability</i>	<i>Hsp70 status</i>		
			MFI at 37°C	MFI at 43°C	Ind. (fold)
Low Zn (10)	11.8 \pm 0.5	1.21 \pm 0.02	9.4 \pm 1.1	26.5 \pm 4.8	2.7 \pm 0.4
High Zn (10)	13.1 \pm 1.1	1.39 \pm 0.02	16.7 \pm 1.5	97.4 \pm 14.3	5.9 \pm 0.7
P	0.068	< 0.0001	0.001	0.001	0.001

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48 **Table 2 Relationship between the zinc status and the stress response.** Subjects

49 were grouped according to their zinc availability (high Zn > 1.30 \geq low Zn). Data were

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52 obtained as described in Subjects and methods and shown as mean \pm SEM. Basal (37°C) and

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55 heat-induced Hsp70 (43°C) levels are indicated as the mean fluorescence intensity (MFI),

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58 Hsp70 inducibility (Ind.) is given as the 43°C/37°C ratio. p values were calculated using the

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61 Student's independent samples t-test.
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4 **1 Figure legends**

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8 **3 Figure 1** Differential stress response in different lymphocyte populations

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10 **4 (A)** Scatterplot distribution of lymphocytes in the forward vs. side scattergram. **(B)**

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12 **5** Differentiation of CD3⁻ (R3) and CD3⁺ (R2) lymphocyte populations using an anti-CD3/APC

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14 **6** fluorescence. **(C)** Histograms of basal (37°C) and heat-induced (43°C) Hsp70 levels in total,

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16 **7** CD3⁺ and CD3⁻ populations by depicting the anti-Hsp70/FITC fluorescence. The figure is a

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18 **8** representative of 3 independent experiments.

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24 **10 Figure 2** Relationship between basal and heat-induced Hsp70 levels in CD3⁺ and

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26 **11** CD3⁻ lymphocyte populations from old donors.

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28 **12** PBMC were obtained from healthy elderly patients (n = 20). Hsp70 levels were

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30 **13** expressed as the mean fluorescence intensity (MFI). Observed heat-induced (43°C) vs. basal

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32 **14** (37°C) Hsp70 MFIs were plotted from CD3⁺ **(A)** and CD3⁻ **(B)** lymphocyte populations of the

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34 **15** same individual. Bivariate linear correlations were calculated using the Pearson's algorithm

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36 **16** and the parameters are indicated on the charts.

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43 **18 Figure 3** Hsp70 status of lymphocytes from individual healthy old subjects before

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45 **19** and after clinical zinc supplementation.

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47 **20** PBMC were obtained from the same patients (n = 7) before and after a 48-day oral

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49 **21** zinc supplementation (10 mg pure zinc/day) and the experiments were performed as described

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51 **22** in Materials and Methods. Individual basal (37°C, **A**) and heat-induced (43°C, **B**) Hsp70

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53 **23** levels are indicated as the mean fluorescence intensity (MFI), Hsp70 inducibility **(C)** is given

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55 **24** as the 43°C/37°C ratio.

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60 **25**

1 **Figure 4** Hsp70 status of different lymphocyte populations from healthy old subjects
2 before and after clinical zinc supplementation.

3 PBMC were obtained from the same patients (n = 7) before and after a 48-day oral
4 zinc supplementation (10 mg pure zinc/day) and the experiments were performed as described
5 in Materials and Methods. Basal (37°C, **A**) and heat-induced (43°C, **B**) Hsp70 levels were
6 expressed as the mean fluorescence intensity (MFI), Hsp70 inducibility (**C**) is given as the
7 43°C/37°C ratio. Bars represent the mean + SEM of the total (All), the CD3+ and the CD3-
8 lymphocyte populations, respectively. p values were calculated using the Student's paired
9 samples t-test and levels of significance are as follows: * denotes p < 0.05, ** denotes p <
10 0.005, *** denotes p < 0.001.

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12 **Figure 5** Hsp70 status of different lymphocyte populations from healthy old subjects
13 undergoing or not clinical zinc supplementation.

14 PBMC were obtained from patients not supplemented (Non-suppl., n = 10) or
15 supplemented (Zn-suppl., n = 10) with zinc (10 mg pure zinc/day for 48 ± 2 days) and the
16 experiments were performed as described in Subjects and methods. Basal (37°C, **A**) and heat-
17 induced (43°C, **B**) Hsp70 levels were expressed as the mean fluorescence intensity (MFI),
18 Hsp70 inducibility (**C**) is given as the 43°C/37°C ratio. Bars represent the mean + SEM of the
19 total (All), the CD3 positive (CD3+) and the CD3 negative (CD3-) lymphocyte populations,
20 respectively. p values were calculated using the Student's independent samples t-test and
21 levels of significance are as follows: * denotes p < 0.05, ** denotes p < 0.01.

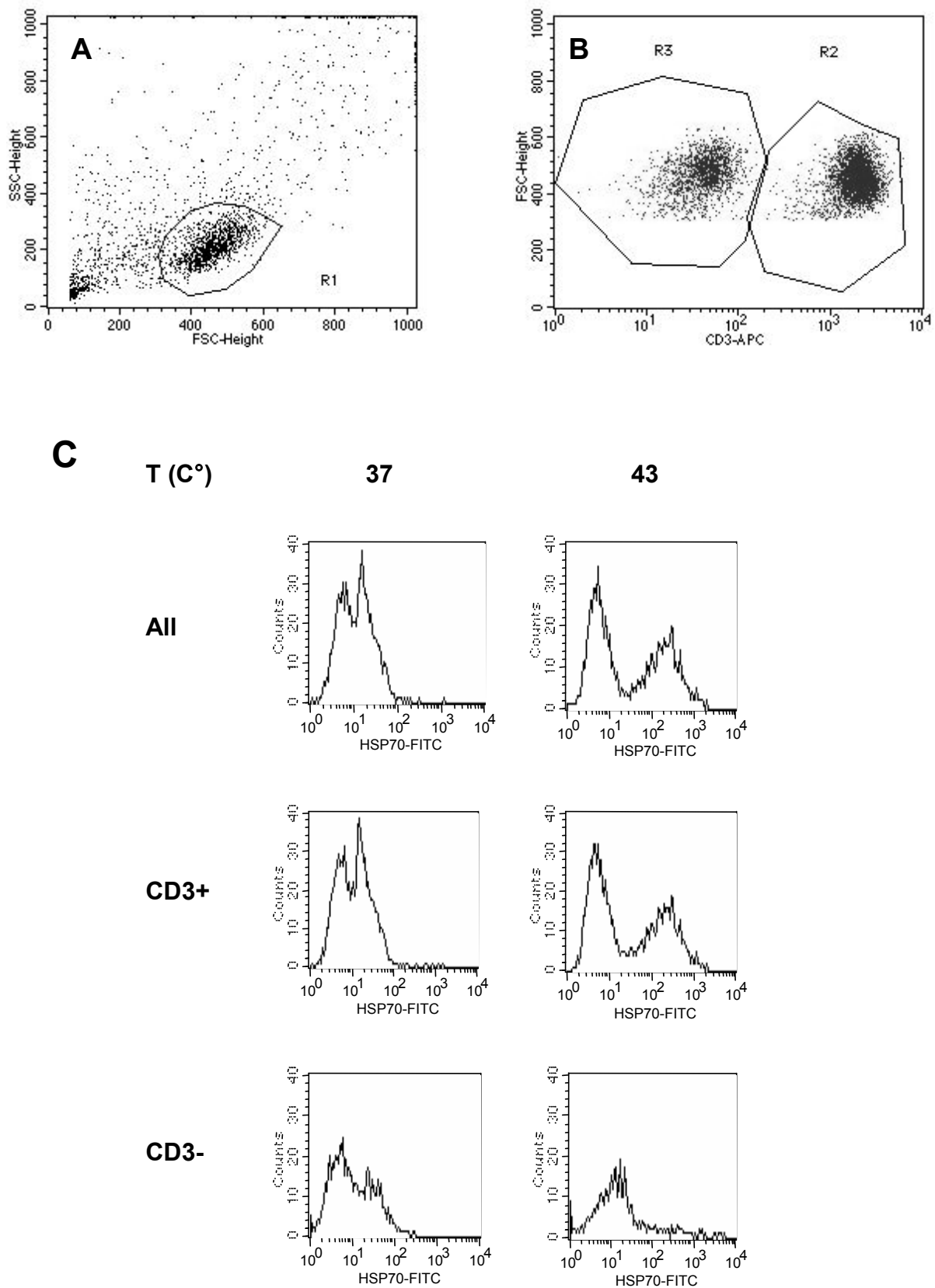
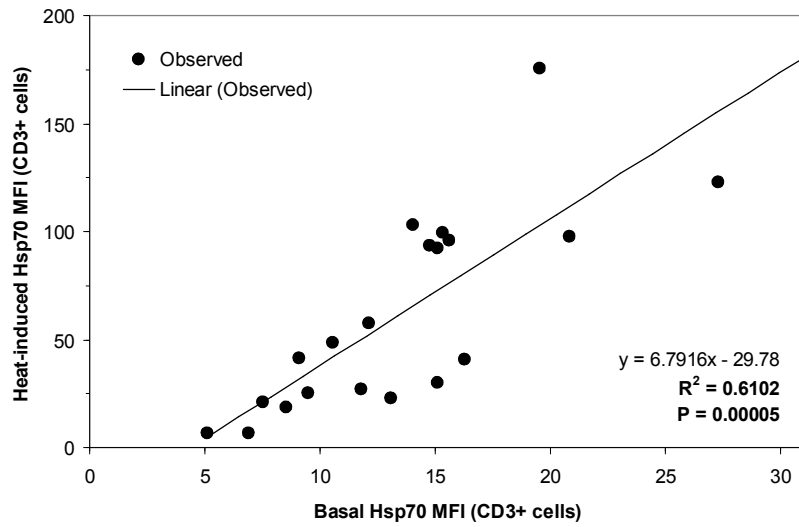
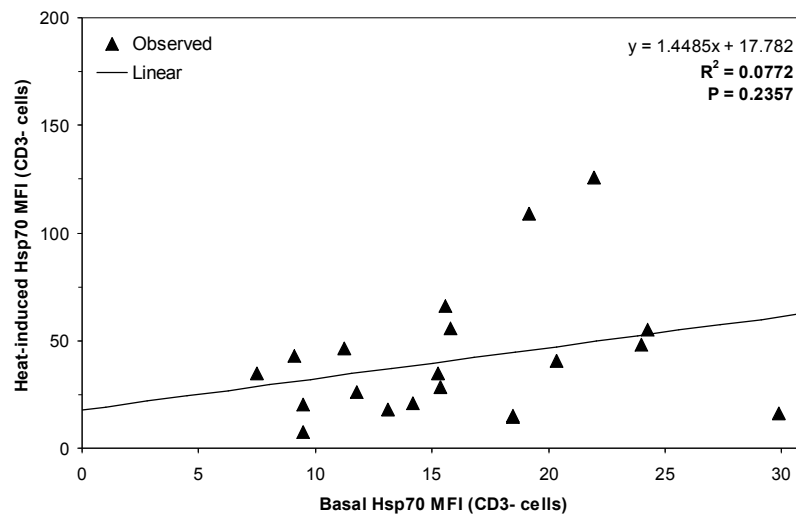


Figure 1 of Putics et al.

A**B****Figure 2 of Putics et al.**

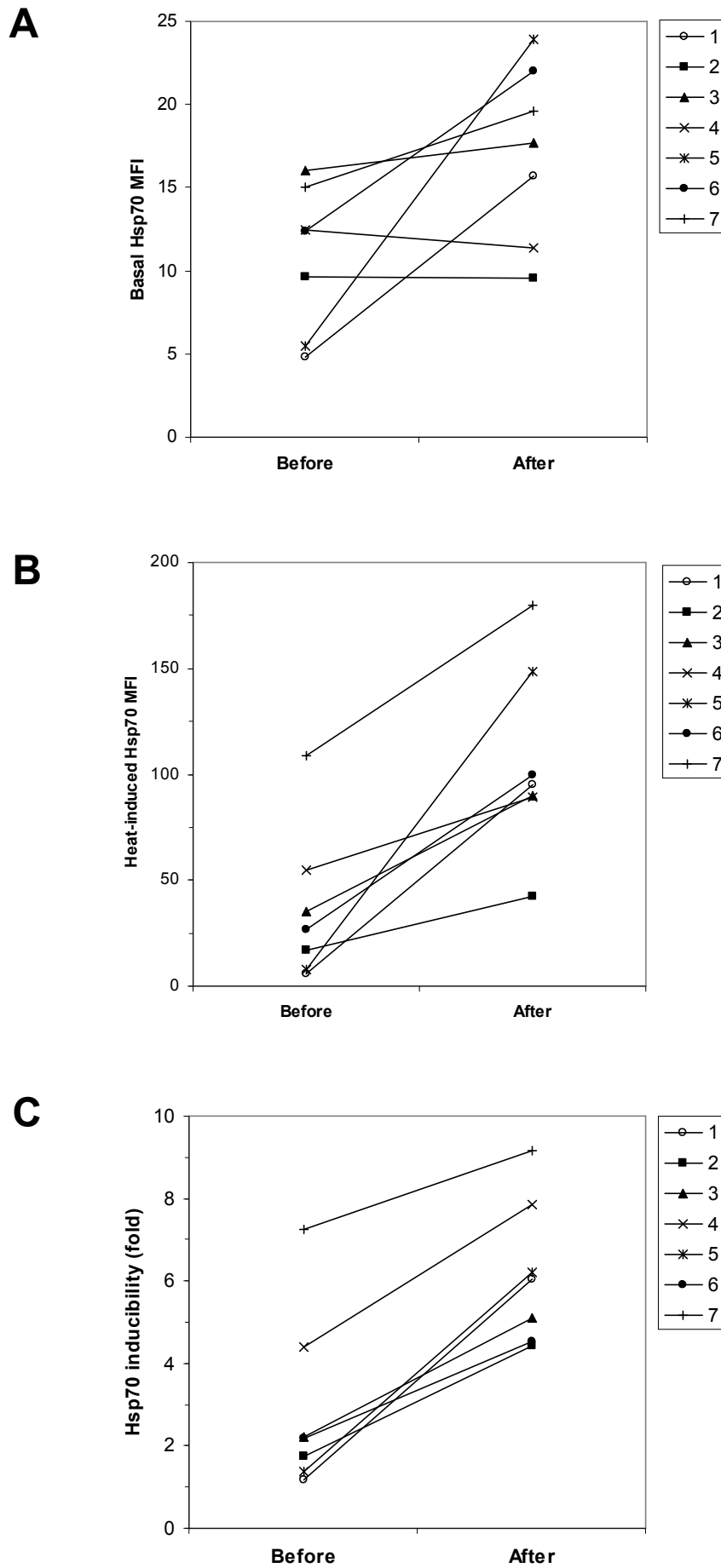


Figure 3 of Putics et al.

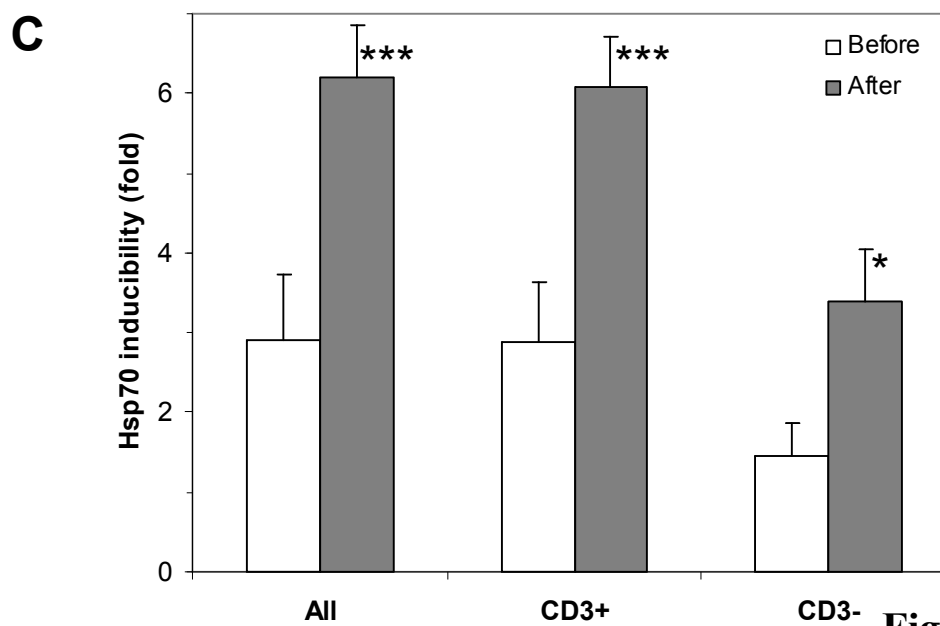
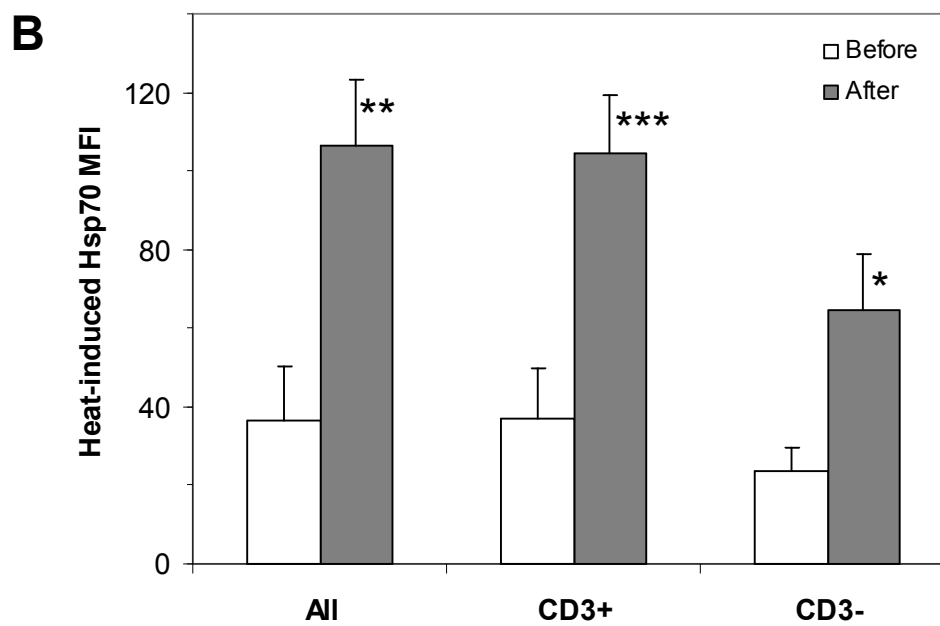
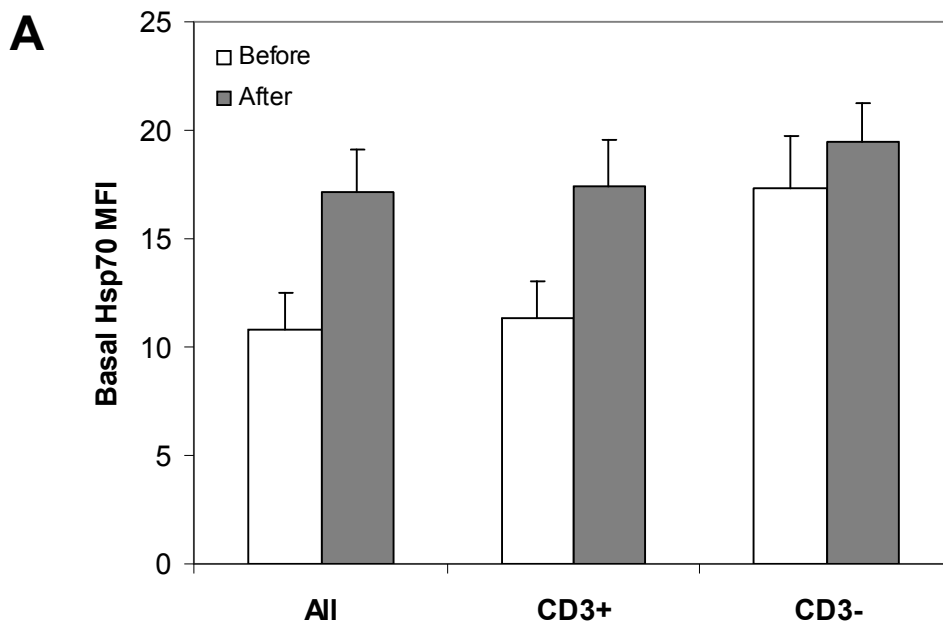


Figure 4 of Putics et al.

