Commentary to: ‘A comparison of two commercially available ELISA methods for the quantification of human plasma heat shock protein 70 during rest and exercise stress’ by Lee et al. 2015

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The study of Lee et al. (2015) aims to compare the capacity of two commercially available ELISA kits (ENZ-KIT and EKS-715) to detect extracellular Hsp70 (eHsp70) in healthy males within an age range of 19 to 27 years before and after exercise under heat- and hypoxic-stress conditions. We have recently published a paper (Breuninger et al. 2014) entitled: ‘Quantitative analysis of liposomal heat shock protein 70 (Hsp70) in the blood of tumor patients (serum and plasma) using a novel lipHsp70 ELISA’ which shows the validation of a newly developed lipHsp70 ELISA. In this study, circulating Hsp70 levels were quantified in the serum and plasma of healthy human individuals ($n=114$) and compared with that of patients bearing tumors of different entities such as squamous cell carcinoma of the head and neck (SCCHN, $n=23$), non-small cell lung carcinoma and small cell lung carcinoma (NSCLC and SCLC, $n=22$), colorectal carcinoma ($n=44$), pancreatic carcinoma ($n=46$), glioblastoma ($n=30$), and hematological malignancies ($n=32$). Herein, we aim to compare the results derived by the different commercial Hsp70 ELISAs and the novel lipHsp70 ELISA which was established and patented in 2014.

Lee et al. investigated the eHsp70 levels in the plasma of 21 young males (mean age around 22 years) before and after exercise under heat/hypoxic conditions under highly standardized conditions using two different commercially available

Hsp70 ELISAs (EKS-715 and ENZ-KIT). In their study, EDTA plasma was obtained from physically active, non-smoking humans after an overnight fasting period, and following exercise after reaching peak oxygen consumption. The plasma was diluted at different ratios ranging from 1:4 (as recommended) up to 1:16 to measure data within the standard curve. The measurements were conducted either at 450 nm (EKS-715) or at 495 nm (ENZ-KIT) to narrow down the comparability between the two assays. The authors postulate that serum appears to yield lower basal values than plasma (Lee et al. 2015) in a small panel of individuals. When measuring plasma and serum samples of 114 healthy human volunteers with the lipHsp70 ELISA, no significant differences between serum and plasma could be detected. Following the recommendations of a statistician, we comparatively analyzed the serum and EDTA plasma of 67 males and 47 females in a wider age range of 20 to 74 years (42.9 ± 14.6 years). By using our novel established lipHsp70 ELISA assay, no significant differences in the circulating Hsp70 levels were observed in the different age groups, after food intake of the donors, repeated freezing and thawing procedures, moderate hemolysis, or if serum or plasma samples were used for the assay. Furthermore, and also in contrast to the study of Lee et al., a dilution ratio of 1:5 could be employed for all tested samples of healthy individuals and tumor patients. In our study, this identical dilution was used to control eventually occurring matrix effects of the serum/plasma samples. In accordance to the measurements using the EKS-715 ELISA assay, our measurements were also conducted at a wavelength of 450 nm and corrected by absorbance at 570 nm.

Presently, two major pathways are discussed to explain the secretion of Hsp70 into the serum in healthy individuals and patients. On the one hand, Hsp70 can be released by dying cells as a free protein and on the other hand, a major part of Hsp70 in the circulation is bound to lipid vesicles, (most likely

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exosomes) which can be actively released by living cells (Gastpar et al. 2005; DeMaio 2011). Therefore, the reliability of the novel lipHsp70 ELISA assay was validated by spiking experiments using free and/or liposomal Hsp70. A comparison of the recovery rate of spiked free or liposomal Hsp70 revealed a significantly better recovery of both forms of Hsp70 by the lipHsp70 ELISA compared to that of a commercial R&D ELISA (Breuninger et al. 2014). Repeated measurements of spiked recombinant Hsp70 and liposomal Hsp70 demonstrated a high reliability of the lipHsp70 ELISA ($101 \pm 3 / 76 \pm 5$ %) for the lipHsp70 ELISA but not for the commercial Hsp70 ELISA ($57 \pm 3 / 7 \pm 1$ %). The work of Lee et al. (2015) did not include spiking experiments using free and/or lipid-bound Hsp70 and thus data derived by the commercial Hsp70 ELISAs cannot be used for quantitative analysis (Whitham and Fortes 2006).

The mean serum levels of 114 healthy individuals measured with the lipHsp70 ELISA was 6.4 ± 2.7 ng/ml. This value is significantly higher than that measured with the R&D (2.8 ± 1.3 ng/ml in 114 out of 114), EKS-715 (3.57 ± 2.68 ng/ml in 6 out of 32), and the ENZ-KIT (1.54 ± 3.19 ng/ml in 32 out of 32) ELISA for healthy human individuals under resting conditions. The improved sensitivity (0.007 ng/ml) of the ENZ-KIT ELISA was achieved by an alkaline phosphatase conjugate which binds to a signal amplification substrate and thus results in an enhanced color production at low analyte concentrations. However, since liposomal Hsp70 is neither detected by the EKS-715 nor by the ENZ-KIT ELISA, the increased sensitivity did not reflect elevated Hsp70 serum levels. The intra-assay precision of EKS-715, ENZ-KIT, and lipHsp70 ELISAs was 2.6, 4.1, and 5.2 to 8.1 %, respectively, and the inter-assay precision was 4.9, 6.2, and 10.9 %, respectively. Depending on the amount of the detected circulating Hsp70 in the serum/plasma, the working range of EKS-715, ENZ-KIT, and lip Hsp70 ELISAs also differed (0.2–12.5, 0.039–5, and 0.36–17.41 ng/ml, respectively).

The paper of Lee et al. (2015) claims that elevated Hsp70 levels are found in the circulation of patients with autoimmune and inflammatory diseases (Luo et al. 2008; Schick et al. 2004; Najafizadeh et al. 2015) and thus could act as a biomarker for these diseases, although none of the two commercial ELISAs were tested with serum of patients. Furthermore, it is also well known that significantly elevated levels of Hsp70 in the blood are also detectable in the blood of tumor patients (review in: Pockley et al. 2014).

Taken together, the lipHsp70 ELISA compared to the commercially available Hsp70 ELISAs EKS-715, ENZ-KIT, and R&D ELISA provides a robust tool for a quantitative measuring of both free and lipid-bound Hsp70 in the serum and plasma of healthy individuals and patients. The significantly

elevated levels of Hsp70 in the serum of tumor patients as determined with the lipHsp70 ELISA in the peripheral blood might provide a useful tool for the detection of tumors, inflammatory diseases, and for monitoring the clinical outcome of a certain disease in the serum and plasma of patients.

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Conflict of interest The new LipHsp70 ELISA which was established, published (Breuninger et al. 2014), and patented in the laboratory of Gabriele Multhoff is in direct competition with the ENZO Hsp70 ELISA kits.

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