Long-term endurance training increases serum cathepsin S levels in healthy female subjects

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Abstract

Background Circulating cathepsin S (CS) has been associated with a lower risk for breast cancer in a large Swedish cohort. Long-term physical activity has been shown to have beneficial effects on the development of various cancer subtypes, in particular breast and colorectal cancers. The aim of this study was to investigate the effect of long-term endurance sport on CS levels in females.

Material and methods Thirty-six of 40 subjects completed the study. Subjects were told to increase their activity pensum for 8 months reaching 150 min/week moderate or 75 min/week intense exercise. Ergometries were performed at the beginning and the end of the study to prove/quantify the performance gain. Blood samples were drawn at baseline and every 2 months. Serum CS levels were measured by ELISA. To analyse the change and the progression of CS, Wilcoxon rank sum and Friedman tests were used.

Results The sportive group (performance gain by > 4.9%) showed a significant increase of CS levels from 3.32/2.73/4.09 to 4.00/3.09/5.04 ng/ml ($p = 0.008$) corresponding to an increase of 20.5%.

Conclusions We could show a significant increase of circulating CS levels in healthy female subjects induced by long-term physical activity. CS, occurring in the tumour microenvironment, is well-known to promote tumour growth, e.g. by ameliorating angiogenesis. However, the role of circulating CS in cancer growth is not clear. As physical activity is known as a preventive intervention, in particular concerning breast and colorectal cancers, and long-term physical activity leads to an increase of CS levels in female subjects, circulating CS might even be involved in this protective effect.

Trial registration Clinical trial registration: NCT02097199

Keywords Breast cancer · Cathepsin S · High-sensitivity CRP · Interleukin-6 · Physical activity

Background

The influence of sports on atherosclerosis [1], inflammation and calcification [2] biomarkers or the lipid profile [3] and its beneficial effect on cardiovascular disease are well-known, and the protective effect of physical activity against the development of malignant diseases is also well established. Recently, a meta-analysis of 126 studies showed that physical activity according to the WHO recommendations leads to a decrease of cancer incidence of 7% for colorectal and breast cancers [4]. Another study focused solely on breast cancer and indicated that > 7-h walking per week reduced the risk for breast cancer by 14% [5]. Conversely, longer leisure time spent sitting was associated with a higher incidence of breast cancer, multiple myeloma and ovarian cancer in women [6]. However, the biochemical changes that underlie these...
beneficial effects on the incidence of various cancer types are not fully understood.

In general, cysteine cathepsins are suspected to play a key role in tumour growth. They are said to promote tumour progression by initiating a proteolytic cascade leading to the activation of MMPs (matrix metalloproteinases), uPA (urokinase-type plasminogen activator) and plasminogen [7]. Furthermore, several in vitro studies pointed out the role of cathepsin S (CS) in prompting tumour angiogenesis which is additionally promoted by matrix-derived angiogenic factors like the pro-angiogenic gamma-2 fragment of laminin-5 [8, 9].

As a lysosomal serine protease, CS is predominantly expressed in antigen-presenting cells. It participates in the degradation of the invariant or light chain that prevents the loading of the antigen into the complex. Hughes et al. [10] demonstrated that extracellular CS activity levels (together with interleukin (IL)-1β) might act as surrogate for lysosomal damage and particle-induced inflammation in vitro. Clinically, CS has been shown to be expressed in a great number of malignant tumours such as colon, pancreatic and hepatocellular cancers [11–13]. In vitro experiments showed its involvement in cancer cell migration and degradation of the extracellular matrix therefore promoting tumour genesis and enhancing angiogenesis [8, 14]. On the contrary, knockout of CS leads to decreased invasion, tumour growth and angiogenesis and an increase in apoptosis [9, 15, 16]. The main sources for CS are from tumour-associated macrophages (TAMs) and the cancer cells itself. However, the role of systemic CS is not yet fully understood. Lately, the ‘Swedish Mammography Cohort’ study [17] could show that higher levels of systemic CS (and high-sensitivity C-reactive protein (hsCRP)-adjusted CS) are associated with a lower risk of breast cancer. It was therefore the aim of this prospective observational study to investigate the influence of long-term physical activity on serum CS levels in female subjects with proven performance gain of > 4.9% over 8 months of training.

Material and methods

Population

We could show in a previous work [18] that long-term physical activity leads to a significant increase of CS serum levels and a decrease of IL-6 and hsCRP. The present study uses data of the female participants of the mentioned study [18], which originally focused on CS and cardiovascular disease. However, in contrast to this study, for statistical analysis, the threshold for significant performance gain has been set at 5% as described in the “Statistical analysis” section to deliver balanced groups of 18 participants in each group. The participants were recruited at the Medical University of Vienna, Department of Internal Medicine II. The inclusion criteria for the analysis of the present study were female sex, age between 30 and 65 years and physical ability to perform endurance exercise. Exclusion criteria were age < 30 or > 65 years, no ability to perform endurance exercise and current oncologic or infectious disease (anamnestic or increased inflammation parameters at baseline).

Measurement of anthropometric data and bicycle stress test (ergometry)

After detailed anamnesis and physical examination including the measurement of height, weight, body water, body muscle mass and body fat (with a diagnostic scale, Beurer BG 16, Beurer GmbH, Ulm, Germany), the subjects had to perform a bicycle stress test (ergometry) at the beginning of the study to define their performance level and to calculate their individual training pulse/target heart rate (using the Karvonen formula with an intensity level of 65–75% for moderate and 76–93% for vigorous intensities). The subjects were let to decide the kind of physical activity/sports; however, they were asked to perform at least 75 min/week of vigorous- or 150 min/week of moderate-intensity endurance training (or a mixture; strength training was allowed but not mandatory) within the calculated training pulse. Moderate intensity was defined as slow cycling/swimming, skating, gymnastic or northern walking; vigorous intensity was defined as playing soccer, tennis, basketball, quick cycling or jogging. A second ergometry was performed at the end of the study (after 8 months) to prove and also quantify exactly and objectively the change/gain in performance. Therefore, we relinquished the leading of a training protocol. The bicycle stress tests were always ECG-monitored and performed with the same system (Ergometer eBike comfort, GE Medical Systems, Freiburg, Germany) starting with 25 W and increasing every 2 min by 25 W (according to the protocol of the Austrian Society of Cardiology which is equal to the guidelines of the European Society of Cardiology). Blood pressure and heart rate were taken every 2 min. Subjects were told to cycle with 50–70 rpm until exhaustion occurred. The target performance was calculated using body surface (calculated according to the DuBois formula: body surface \( [m^2] = 0.007184 \times \text{height}^{0.725} \times \text{weight}^{0.425} \)) [19], sex and age. An individual target performance of 100% represents the performance of an untrained collective. The study was carried out in adherence to the declaration of Helsinki and its later amendments. The protocol has been approved by the ethical commission of the Medical University of Vienna, Austria, and informed consent was obtained from all subjects.

Laboratory analysis

Blood samples were drawn in a not starving state for five times: at the beginning of the study (baseline levels) and every 2 months.
(2/4/6/8 months). All blood samples were taken after 10 min of still lying from an arm vein with a tube/adapter system. Samples for the determination of routine laboratory parameters were analysed immediately after drawing. Samples for the determination of CS (Cusabio Biotech, MD 20740, USA) were centrifuged and frozen immediately after drawing. The ELISA analysis was performed according to the manufacturer’s instructions. The CS assay had a total coefficient of variation (CV) of about 6%; β-estradiol, hsCRP and IL-6 levels were analysed in the course of the routine laboratory analysis.

**Statistical analysis**

Statistical analysis was accomplished using SPSS 20.0. Continuous and normally distributed data is described by mean ± standard deviation (SD). Not normally distributed data is described by median/25th quartile/75th quartile. As it was expected that not all of the subjects would reach an adequate performance gain during the observation period, we defined a minimum threshold of 5% performance gain as significant and divided the study population into two groups: group 1 (performance gain ≤ 4.9%) and group 2 (performance gain > 4.9%). To investigate the difference between baseline and 8-month levels of β-estradiol, CS, hsCRP and IL-6, we used a non-parametric test for two related samples (Wilcoxon rank sum test). To investigate the significance of the tendency of the progression (baseline/2/4/6/8 months), we used a non-parametric test for five related samples (Friedman test). All tests were performed in accordance with two-sided testing, and p values ≤ 0.05 were considered significant.

**Results**

Routine laboratory data at baseline of both groups are shown in Table 1. Except for GOT (glutamat-oxalacetat-transaminasis), there were no significant differences and the homogeneity of the both groups at baseline is represented. Anthropometric data is shown in Table 2. There was neither significant difference in age, BMI, body muscle mass/fat/water nor in SBP, DBP or heart rate at baseline. The mean performance gain in the non-sportive group 1 was −0.82 ± 5.33% and in the sportive group 2 12.35 ± 5.52%.

When correlating baseline anthropometric and laboratory data with CS, we found a positive correlation of CS with lipoprotein(a) (p = 0.018; correlation coefficient 0.388) and serum calcium (p = 0.012; correlation coefficient 0.409) and a negative correlation with β-estradiol (p = 0.025; correlation coefficient −0.368) and chloride (p = 0.022; correlation coefficient −0.374). There was a statistically not significant positive correlation of CS with hsCRP (p = 0.640; correlation coefficient 0.308).

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**Table 1 Routine laboratory data at the baseline of both groups**

|                      | Group 1 (performance gain ≤ 4.9%) | Group 2 (performance gain > 4.9%) | p value |
|----------------------|-----------------------------------|-----------------------------------|---------|
| n                    | 18                                | 18                                |         |
| Erythrocytes (T/l)   | 4.38 ± 0.27                       | 4.42 ± 0.31                       | 0.690   |
| Haemoglobin (g/dl)   | 12.97 ± 0.91                      | 13.01 ± 1.13                      | 0.923   |
| Haematocrit (%)      | 37.81 ± 2.07                      | 37.49 ± 2.42                      | 0.673   |
| Thrombocytes (g/l)   | 272.61 ± 53.41                    | 245.74 ± 62.69                    | 0.170   |
| Leukocytes (g/l)     | 6.84 ± 1.74                       | 6.31 ± 1.58                       | 0.344   |
| Na (mmol/l)          | 140.61 ± 1.75                     | 141.11 ± 1.49                     | 0.361   |
| K (mmol/l)           | 4.21 ± 0.24                       | 4.08 ± 0.29                       | 0.153   |
| Cl (mmol/l)          | 101.00 ± 1.81                     | 101.53 ± 2.27                     | 0.443   |
| Ca (mmol/l)          | 2.30 ± 0.10                       | 2.31 ± 0.11                       | 0.714   |
| Mg (mmol/l)          | 0.83 ± 0.05                       | 0.83 ± 0.04                       | 0.912   |
| Creatinine (mg/dl)   | 0.79 ± 0.10                       | 0.74 ± 0.11                       | 0.103   |
| Urea (mg/dl)         | 12.47 ± 3.18                      | 22.43 ± 31.25                     | 0.188   |
| Uric acid (mg/dl)    | 4.52 ± 1.23                       | 3.92 ± 0.79                       | 0.082   |
| Albumin (g/l)        | 43.66 ± 2.52                      | 44.16 ± 1.77                      | 0.486   |
| Lipase (U/l)         | 33.83 ± 10.68                     | 36.16 ± 10.47                     | 0.508   |
| Cholin esterasis (kU/l) | 7.03 ± 1.28                     | 7.40 ± 1.36                       | 0.409   |
| GOT (U/l)            | 26.56 ± 9.44                      | 19.63 ± 5.13                      | 0.008   |
| GPT (U/l)            | 22.33 ± 8.89                      | 18.11 ± 7.07                      | 0.117   |
| γ-GT (U/l)           | 16.44 ± 9.13                      | 13.42 ± 4.67                      | 0.210   |
| Triglycerides (mg/dl)| 115.17 ± 98.34                    | 101.95 ± 53.50                    | 0.612   |
| Cholesterol (mg/dl)  | 207.56 ± 43.63                    | 199.95 ± 38.28                    | 0.576   |
| HDL-cholesterol (mg/dl) | 67.89 ± 24.27                   | 67.21 ± 14.84                     | 0.918   |
| LDL-cholesterol (mg/dl)| 118.49 ± 37.07                | 112.35 ± 36.83                    | 0.616   |
| HbA1c (rel.%)        | 5.46 ± 0.97                       | 5.19 ± 0.28                       | 0.248   |

Data is given as mean ± standard deviation

GOT glutamat-oxalacetat-transaminasis, GPT glutamat-pyruvat-transaminasis, γ-GT γ-glutamyltransferasis, HDL-cholesterol high-density lipoprotein cholesterol, LDL-cholesterol low-density lipoprotein cholesterol

As shown in Table 3, there was a significant increase of CS levels from 3.32/2.73/4.09 to 4.00/3.09/5.04 ng/ml (p = 0.008) in group 2 corresponding to an increase of 20.5%. There was also a CS increase detectable in group 1 but without statistical significance. Furthermore, we found a significant decrease in β-estradiol in group 2 (from 23.0 to 10.0 pg/ml; p = 0.033) but not in group 1 (from 27.5 to 33.5 pg/ml). The body fat percentage decreased significantly in group 2 from 34.3 to 32.7% (p = 0.033) whereas there was no significant change in group 1 (34.6 vs. 33.5%; p = 0.393). The BMI decreased in group 2 from 26.3 to 25.4 kg/m² (p = 0.802) and also decreased in group 1 from 27.0 to 26.9 kg/m² (p = 0.064). Concerning IL-6, we stated a trend towards a decrease from 2.03/1.50/2.79 to 1.50/0.00/2.84 pg/ml but without significant difference (p = 0.170), probably due to the low number of participants.
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In this study, we could show that a gain in physical fitness due to long-term endurance sports leads to a significant increase in circulating CS serum levels in healthy female subjects. The protective effect was more pronounced for breast and colorectal cancers.

However, the biochemical mechanisms mediating this protective effect are not fully understood. In particular, there is hardly any data available concerning the influence of long-term physical activity on CS serum levels, although CS, which is present in the tumour microenvironment, has been shown to play role in cancer development.

The basic role of CS in inflammation is widely known: CS is a lysosomal enzyme which is expressed by antigen-presenting cells (macrophages, microglia and dendritic cells). It maintains the cleavage of lip1 leaving a part of it (the CLIP—class II-associated invariant chain peptide) associated with the major histocompatibility complex (MHC II). Thus, CS facilitates the dissociation of CLIP from MHC II and enables the loading of the selected antigen by the MHC II. Concerning cancer growth, it has been shown that tumour-associated macrophages are the main source of CS in the tumour environment. By facilitating neovascularisation in the microenvironment of the tumour, CS acts as pro-tumourigenic factor [20]. The pro-tumorigenic properties of CS in the tumour microenvironment have been further described by Ward et al. [21] with a colorectal carcinoma xenograft mouse model. CS did not only exert pro-angiogenic effects on the extracellular matrix but also additionally led to a degradation of antiangiogenic factors. These effects were reversible by treatment with the CS inhibitory antibody, Fsn0503.

However, results from the Swedish Mammography Cohort study [17] showed that higher levels of systemic/circulating CS (and hsCRP-adjusted CS) are associated with a lower risk for breast cancer. The inverse association between CS and breast cancer risk, when comparing the top to the bottom tertile, was significant with an OR (odds ratio) of 0.46. Up to now, CS was in the focus of many in vitro studies investigating its tumour-promoting effects on different cancer cell lines. In the tumour microenvironment, CS is mainly secreted by TAMs and tumour cells promoting tumour growth, angiogenesis and invasion [8, 9]. On the other hand, the role of serum CS is yet unclear. In obese men, higher CS serum levels were associated with higher pro-inflammatory cytokines like IL-6 and an increase of hs-CRP [22]. Additionally, higher CS serum levels were associated with decreased insulin sensitivity and higher mortality in two cohorts of elderly people [23, 24]. Recently, it was shown that CS serum levels were higher in patients with gastric cancer and that serum CS levels correlated with the tumour volume [25]. No data for breast cancer patients regarding this topic exists. However, it is well-established that CS can promote gastric cancer cell migration and invasion [26]. Moreover, in vivo mouse model of multi-stage islet cell carcinogenesis highlighted the role of CS in tumour cell proliferation and angiogenesis [8]. It is clear that the role of secreted CS in the tumour microenvironment is to

**Discussion**

In this study, we could show that a gain in physical fitness due to long-term endurance sports leads to a significant increase in circulating CS serum levels in healthy female subjects.

It is well-established that physical activity exerts a protective effect against the development of different cancer types. Recently, Liu et al. [4] addressed this issue extensively by comparing the results of 126 high-quality epidemiologic studies dealing with this topic. They found a 10% reduction in total cancer risk by comparing the individuals with the highest level of physical activity to individuals doing no sports. Furthermore, the analysis showed that the current WHO recommendations for physical activity (at least 2.5 h/week of moderate intensity or 1.25 h/week of high intensity or a combination) lead to a 7% risk reduction for cancer. The protective effect was more pronounced for breast and colorectal cancers.

However, the biochemical mechanisms mediating this protective effect are not fully understood. In particular, there is hardly any data available concerning the influence of long-term physical activity on CS serum levels, although CS, which is present in the tumour microenvironment, has been shown to play role in cancer development.

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degrade the extracellular matrix thereby facilitating metastasis, angiogenesis and invasion, which is proven by the mentioned studies. The role of CS in healthy people is unclear yet. Circulating CS might have a protective effect on breast cancer regarding the data of the Swedish Mammography Cohort [17]. It is well-established that long-term physical activity can reduce the incidence of cancer, in particular breast cancer. We have shown in the present work that sports can significantly elevate the CS levels in healthy female volunteers which might serve for a possible explanation for the protective effects even though the biochemical changes that might be connected with higher CS levels remain still unknown. Another drawback of this pilot study is its small sample size and that follow-up data regarding the incidence of breast cancer are missing.

Data is given as median (25th/75th) percentile. The significance of the difference between baseline levels of CS, hsCRP and IL-6 and levels after 8 months of training was tested using the Wilcoxon rank sum test; the significance of the tendency of the progression of baseline levels to levels after 8 months was tested using the Friedman test.

It should be mentioned that a (statistically not significant) increase in CS levels has been detected in group 1 too. This might also be a result of increased physical activity in this group even though the extent of the performance gain was much lower compared to group 2. Nevertheless, it cannot be excluded that other factors such as smoking cession or dietary changes might have influenced their levels.

Contrary to CS, we could only find a non-significant sports-mediated incidental decrease of IL-6, probably due to the small study population. IL-6 has been associated with metastatic breast cancer and correlated with poor survival by promoting the growth of tumour-initiating cells and protecting these cells from apoptosis [27]. Furthermore, anti-IL-6 monoclonal antibodies lead to apoptosis in tumours [28]. Consequently, a sports-induced decrease of IL-6 could have similar protective effects on cancer development as anti-IL-6 antibodies.
We also found a significant decrease of $\beta$-estradiol in the sportive group although the levels were taken at an unknown menstrual cycle phase and therefore should be interpreted with caution. Estradiol, which physiologically promotes the proliferation of normal breast epithelium, has also been associated with the development and growth of gynaecological cancers (breast, ovarian and endometrial cancers) by binding to oestrogen receptors $\alpha$ and $\beta$ (ERs $\alpha$ and $\beta$) and inducing neoplastic transformation of human breast epithelial cells [29].

However, these findings may serve as a basis for larger studies to fully evaluate the effect of long-term physical activity on CS serum levels and if CS has an influence on the incidence of cancer.

Conclusion

We could show a significant increase of circulating CS levels in healthy female subjects induced by long-term physical activity and a decrease of $\beta$-estradiol. We also stated a positive correlation of CS with lipoprotein(a) and a negative correlation with $\beta$-estradiol at baseline. CS, occurring in the tumour microenvironment, is well-known to promote tumour growth, e.g. by ameliorating angiogenesis. However, it is not clear which effects circulating CS has on the development of different cancer subtypes. As physical activity is well-known as a preventive intervention, in particular concerning breast and colorectal cancers, and long-term physical activity leads to an increase of serum CS levels, circulating CS might even be involved in this protective effect. The effects seen in this study on $\beta$-estradiol levels have to be interpreted with extreme caution as data on the menstruation cycle of our participants are missing. Taken together, a larger number of participants and a longer follow-up are needed to fully evaluate the effects of decreasing systemic CS levels on the incidence of certain cancer subtypes.

Limitations

The present study has several limitations: First, several other factors that were not controlled might have an influence on circulating serum CS/IL-6/hsCRP levels. Second, the number of subjects was quite low. Third, long-term data on the incidence of breast cancer in this cohort is missing. Fourth, $\beta$-estradiol levels were taken at unknown menstrual cycle phase.

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Author contributions Michael Sponder: study design, clinical investigation, performing bicycle stress tests/follow-up, statistical analysis, manuscript preparation

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Michael Emich: study design

Monika Fritzer-Szekeres: laboratory analysis

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The study was carried out in adherence to the declaration of Helsinki and its later amendments. The protocol has been approved by the ethical commission of the Medical University of Vienna, and informed consent was obtained from all subjects.

Abbreviations $\gamma$-GT, $\gamma$-glutamyltransferase; CLIP, class II-associated invariant chain peptide; CS, cathepsin S; CV, coefficient of variation; DBP, diastolic blood pressure; ECG, electrocardiogram; ELISA, enzyme-linked immunosorbent assay; ERs $\alpha$ and $\beta$, oestrogen receptors $\alpha$ and $\beta$; GOT, glutamat-oxalacetat-transaminisation; GPT, glutamat-pyruvat-transaminisation; HDL-cholesterol, high-density lipoprotein cholesterol; HR, heart rate; hsCRP, high-sensitivity CRP; I; Interleukin; LDL-cholesterol, low-density lipoprotein cholesterol; MHC II; major histocompatibility complex II; OR; odds ratio; SBP, systolic blood pressure; SD; standard deviation; TAMs; tumour-associated macrophages

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