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Original Article

Blood plasma main proteins stability of patients with ductal carcinoma in post-surgery period

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Abstract: Thermodynamic stability of plasma/serum major proteins of 14 adult women with breast cancer (BC) without any distant metastasis in post-surgical period from one month to 5 and 17 years has been studied. It was demonstrated that only a simultaneous increase in thermodynamic stability (T_m) and melting interval (DT_m) of the main fatless albumin fraction by 2° and 3°, respectively, in comparison with the norm, as well as a new weakly expressed shoulder that appeared in range 57-60°C from the low-temperature side of the major peak with T_m = 62°C was a direct sign of the initiation of cancer relapse. Transition of the observed weak shoulder into a clear one with further increase in T_m and DT_m is a direct sign of a metastasis process initiation, and transition of this clear shoulder into an intensive individual peak with T_m = 59 ± 1°C corresponds to the morphological and cytological data that was regarded as BC stage III-IV. In all above cases of norm and BC, the melting enthalpy was equal to 20.5 ± 2.5 J/g.deg per dry biomass, and only redistribution of heat between endotherms was observed. Quantity values of suspected biological oncomarkers are calculated at different stages of secondary cancer. Hence, we can suggest that DSC may be successfully used not only as a safe and fast method for estimation of BC risk factors, but it also gives a possibility to observe the course of treatment to find out relapse stability and to be warned about a relapse in time.

Keywords: Differential scanning calorimeter, breast carcinoma, blood plasma/serum, melting temperature, melting enthalpy

Introduction

After the earlier DSC studies of whole blood and blood plasma/serum of patients with various forms of tumors, it was shown that the obtained curves have complex but reproduced profiles for a given disease, and each disease has its clearly expressed individual character depending on a disease stage [1-4]. Among the peculiarities of the DSC profiles that are shown between the norm and pathology, it should be mentioned an appearance of a new clear endotherm at about 59°C. In case of whole blood of healthy persons, it has been shown that the major endotherm about 70°C corresponds to hemoglobin melting in erythrocyte composition, and the weak one at 62°C corresponds to albumin melting. In case of plasma/serum, the main endotherm (heat absorption peak) at about 62°C corresponds to albumin. The difference of plasma melting profile in comparison with the serum melting profile is the endotherm presence with maximum at about 56°C for LT1 (D) fibrinogen fragment [5] and a more thermolabile part of the major albumin stage [6], and about 90°C for HT2 (E) fibrinogen fragment [5]. As a result of the systematic DSC study of blood plasma of cancer [6-17] and non-cancer [18-25] patients has shown that their thermal spectra (melting curve profiles) give some valuable information about thermodynamic stability of the most abundant proteins, which differ for each different disease form. It is suggested that those changes in protein dynamic structures are specific for each disease, and hence DSC may be used successfully in clinics as a diagnostic screening method [1-3, 6, 11, 12, 14-16]. In the given work, on the basis of DSC investigation, we have tried to find out those slight changes in stability of main blood plas-

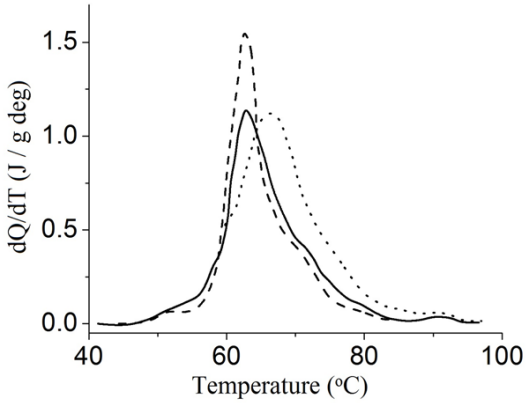

Figure 1. Heat absorption curves as a function of temperature on dQ/dT of 56 years old woman and her daughter blood plasma recalculated per gram dray biomass: 1. Dash line-30 years old healthy daughter. 2. Solid line-the same 42 years old daughter after 12 years. 3. Dot line-56 years old woman with breast cancer, before surgery of two metastasis in node lymph-stage I.

ma/serum proteins, which can give us a possibility to detect relapse of the disease in breast cancer patients at remission stage.

Materials and methods

Blood was collected at the Mammalogy Centre, Institute of Clinical Oncology, in post-surgery period from cancer patients diagnosed according to type, histology, disease stage, nodal involvement, and tumor size. Blood plasma was obtained by blood centrifugation for 10 minutes at 2000 g at 4°C, at presence of K_2-EDTA, and the supernatant without dilution was used for calorimetric study. The DSC measurements have been done immediately after blood collection and centrifugation. The measurements were performed on 42 samples of breast cancer plasma/serum and healthy controls (n = 154) at the Institute of Physics, Tbilisi State University, using a differential scanning microcalorimeter (DSC) for investigation of complex biological systems with sensitivity of 0.1 μW [26]. The operational measuring vessel volume was 125 μl; the dry sample biomass in a vessel was in the range 9.5 to 10.0 mg, the used scanning rate was 1.0° per minute, and the measuring range was from 25°C to 98°C. The accuracy of the temperature measurements was ≤0.05°C. The maximum measuring error in determination of melting enthalpy (DH_m) and heat increment dQ/dT (DC^{max}) was ≤12%. The microcalorimeter (DSC) processor was equipped with relevant software needed for determination of the melting thermodynamic parameters of blood plasma/serum, and the calorimetric curves were plotted and deconvoluted with Origin 9.0. The weight content of total biomass in both normal and cancer samples was determined by dry biomass weight directly in the measuring vessels at 105°C, the ash was determined at 450°C in quartz containers.

We studied development dynamics of the secondary cancer in post-surgery period in 3 groups of female patients with breast carcinoma (BC) without any distant metastases-3 women after ablate of one breast (49, 52, 56 years old, from group 1, in post surgery period from one month to 14 or 15 years), 3 women after lumpectomy (53, 54, 58 years old, from group 2, in post surgery period from one month to 17 years), 8 patients with BC stages I-IV (30-75 years old, from group 3, during 5 years after surgery), 3 adult healthy women who were daughters of 49, 54 and 56 years old patients (group 4), and 154 healthy volunteers (20-75 years old, group 5). The observed curve profiles were similar within each group, and the only difference was in peak intensities, and their endotherm maxima coincide with accuracy 1°C for a given disease stage. Therefore, we presented data of two BC patients per group, namely data of 49 and 56 years old woman from group 1 with the tumor size 22 and 20 mm, respectively, and one 54 years old woman from group 2. In case of this patient, the tumor size was 10 mm, and in cases of other four patients from group 2, the tumor sizes were 7 to 10 mm. We have been monitoring all patients since 1998-2000. Also, we carried out DSC investigation of blood plasma/serum of other 8 patients without metastasis in other organs after lumpectomy during the last 5 years. According to our and clinical data, condition of those patients was stabile-without any replace (data are not presented). They were similar to the data presented in **Figure 1**, dash line.

DSC study of 28 patients' plasma before surgery (size of tumor tissue from 8 to 48 mm) showed a full coincidence of DSC prognosis with clinical data.

Results

In **Figure 1**, there are presented three cases of blood plasma melting: DSC curve of healthy woman 30 and her curve after 12 years without

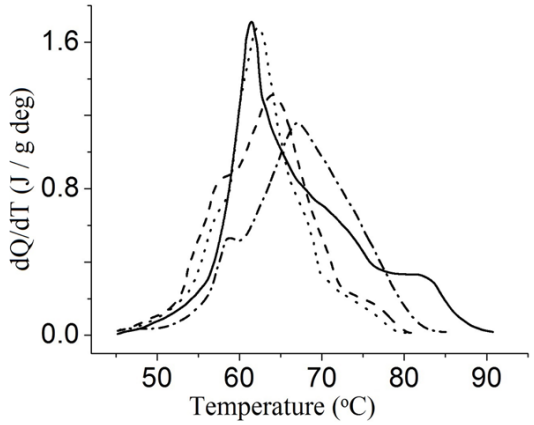

Figure 2. Heat absorption curves as a function of temperature on dQ/dT in case of 56 years old same woman blood serum (BC stage I) after surgical operation. Serum was recalculated per gram biomass. 1. Solid line-in 1-8 years after surgery. 2. Dot line-in 9-10 years after surgery. A risk factor was revealed. 3. Dash line-in 11-12 years after surgery. Clinical diagnosis was lymph node positive, distant metastasis in lungs, stage II. 4. Dot-dash line-13 years after surgery. Clinical diagnosis was lymph node positive, distant metastasis in lungs and liver, stage IV. In all above cases of norm and BC, the melting enthalpy was equal to 20.5 ± 2.5 J/g dry biomass and only redistribution of heat between the endotherms were observed.

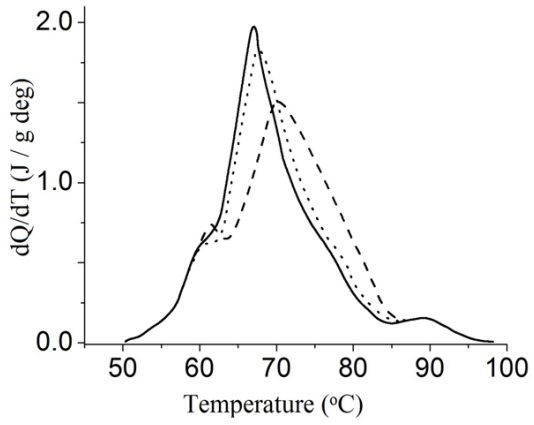

Figure 3. Heat absorption curves as a function of temperature on dQ/dT of the same woman as in **Figures 1** and **2** after treatment. Blood serum is recalculated per gram biomass. 1. Solid line-in 72 hours after blood transfusion and drug treatment (stage IV). 2. Dash line-in 8 days after blood transfusion and drug treatment. 3. Dot line-in 28 days after blood transfusion and drug treatment. She had clinical diagnosis of lymph node positive and distant metastasis.

any detected rigid tissue mass in her mammary glands, and her 56 years old mother with ductal carcinoma stage I. In the first case, the peak with intensity dQ/dT = 1.52 J/g.deg and maximum at around 62 ± 0.1°C and ΔT = 7.2 ± 0.2°C is mainly associated with the albumin fatless fraction. The shoulder in the temperature range 50-56°C and a week peak at 91.5 ± 0.2°C correspond to melting of LT1 (D) and HT2 (D) fragments [5] plus a more thermolabile part of the major albumin stage [6], and E fibrinogen fragment [5] (dash line). Correspondingly, the second curve profile of 42 years old woman without any detected rigid tissue mass in her soft breast (solid line) resembles the profile of the first curve (norm). However, there is also a difference-a weakly expressed shoulder is observed in the temperature range 56-59°C, which is a risk factor for developing of breast cancer BC (risk), according to the work [8, 15]. The dot curve corresponds to melting of plasma from 56 years old woman with ductal carcinoma with two metastases in node lymph, stage I, before surgical operation. As it is shown from the curve, the shoulder at around 59-61°C is more significantly expressed than in the curve (dot line) of her 42 years old daughter with risk of carcinoma development. However, after 3 years, her curve profile and dQ/dT endotherm of the main stage approximated to the norm, and the risk of cancer development reduced further. We suppose that the reduced BC rick is connected with increased consumption of fruits, vegetables and green tea which contain antioxidants (the curve is not presented) [26]. In all above cases of norm and BC, the melting enthalpy was equal to 20.5 ± 2.5 J/g dry biomass.

Figure 2 presents DSC melting curves of blood plasma of 56 years old woman with ductal carcinoma stage I during 14 years after surgical operation. As it is seen from the presented calorimetric curves, there were no significant changes in the melting profile during the first eight years after surgery (see **Figure 1**, dash line). During following 9-10 years, the curve profile has gradually changed. In particular, the main fraction of albumin that melts at 62°C in case of norm, shifted to 64°C and the melting curve gradually widened at its half high (DT_m) from 8.5°C to 10.5°C; a weak shoulder appeared in the temperature range 58 C-60°C (dot line), and it become more expressed at the end of 10 years after surgery, and the clinical diagnosis corresponded to stage I at that time.

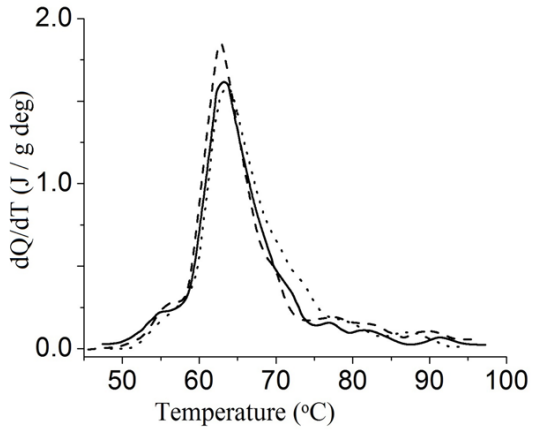

Figure 4. Heat absorption curves as a function of temperature on dQ/dT of woman blood plasma recalculated per gram biomass: Dot line-71 years old woman, in 17 years after a local surgery (BC stage I). The curve did not change during 17 years (data are not present). Dash and solid lines-a healthy daughter of the above patient, when she was 32 (dash line) and 40 (solid line) years old.

Treatment prevents the above mentioned changes in melting curves at presence of risk factors and in case of stage I of the disease. During the following 11-12 years, simultaneously with treatment, the shoulder near 57-60°C become more expressive, and the main fraction albumin melting temperatures (T_m) and DT_m increase by a few degrees. Unfortunately, the patient was found to have lung metastases and clinical diagnosis was stage II by that time. During the following 13-14 years, in the course of treatment, this shoulder transferred to a clear peak with maximum at 59°C ± 1°C, T_m of the main stage increased by 6°C, T_m and DT_m of those peaks continued to increase, and at end of the 13th year they reached 70 ± 1°C and 16 ± 1.5°C, respectively, and some new liver metastases were also found.

It should be noted that after treatment, the curve becomes similar to stage II in about one week, but after this it gradually becomes similar to the curves of the patients with stage IV of the disease in about one or two months (**Figure 3**).

Another picture was observed in case of 54 years old woman (group 2) after local lumpectomy with following breast radiation. Her tumor tissue size was 10 mm (in 1998, diagnosis was BC with one metastasis in lymph node, stage I). She did not receive a course of chemotherapy during 17 years after lumpectomy. **Figure 4** presents melting curve of plasma of the same woman (now she is 71 years old) and her healthy daughter when she was 32 and 40 years old. As it is seen from the present curve, the peak intensity (DC^{max} = dQ/dT = 1.6 J/g deg) and shapes of the present curves are similar with each other.

The deconvolution of blood plasma curve of 62 and 64 years old woman (group 1) with ductal carcinoma stages II and IV (dash-dot line) showed 7 transition stages. Deconvolution of blood plasma of healthy woman was made on the basis of two requirements: (a) melting of major plasma proteins take place independently from each other [18]; (b) clinical data of albumin concentration for particular subject and albumin melting enthalpy [32] were taken into account in deconvolution of plasma curve.

Discussion

In spite of some new and improved methods of early diagnostics, breast cancer remains the most spread disease in women all over the world. Hence, any new methods and approaches for early diagnostics are very important tasks in modern biophysical medicine.

DSC is one of new methods, which gives a possibility to detect cancer at its early stage, on the basis of thermodynamic stability of bio-oncomarkers and their influence on protein dynamic structure, in particular on albumin main fraction in composition of blood plasma/serum of breast cancer patients [2-4, 6-17]. The DSC method is capable to detect cancer with high probability even when no rigid tissue mass with tumor behavior is detected in soft breast tissue with modern mammological methods, or when the rigid tissue is so small that modern mammological methods can give a poor prognosis [29].

DSC method has also some advantages in comparison to X-ray imaging, because DSC is absolutely harmless. This advantage is especially useful for young and pregnant women, as well as in BC post-surgery period as it can be repeatedly used many times during many years. Hormonal analysis with estrogen, progesterone, epidermal growth factor, and other biomarkers (e.g. TNW and TNC) need histological

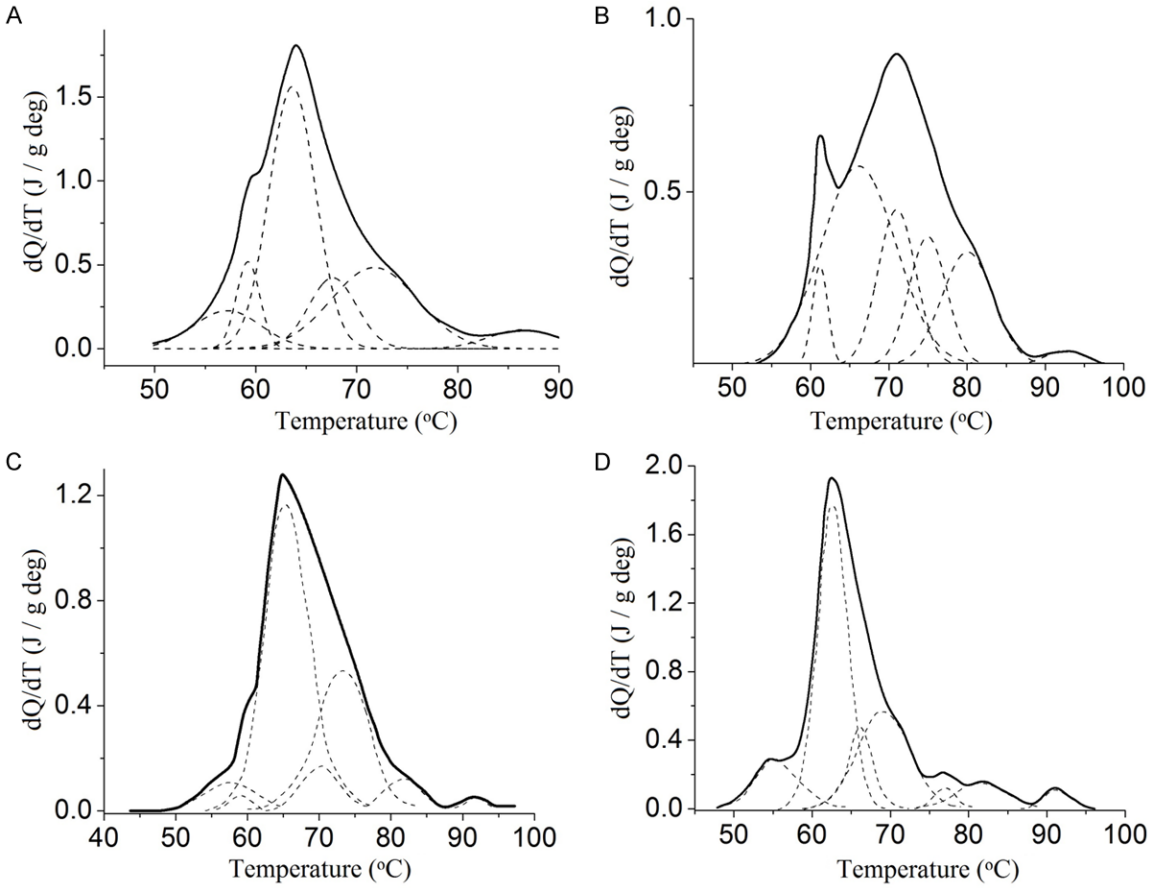

Figure 5. Deconvolution of blood plasma curves in case of BC (A, B) BC risk factor (C), and norm (D). a - Woman 62 years old with BC stage II; b - The same women after 2 stage years stage IV (14 years after surgery); c - A 40 years old daughter of the same women, a risk factor is shown; d - A healthy woman 32 years old.

test and immunoblot analysis, respectively, and both of them need samples from cancer tissues [30]. In case of DSC analysis, it is possible to test BC with fresh collected blood plasma, and the result is obtained in 20-120 minutes.

In the given work, we carried out a systematic investigation of patients with breast cancer during 5-17 years. DSC study has been performed in every 2-3 months, and we can say that the simultaneous appearance of a weak shoulder in the temperature range 58-60°C and increase in T_m and DT_m of albumin fatless fraction by 2-3°C and 8.5-11°C, respectively, in comparison to the norm are signals of relapse. The increase in the albumin main stage T_m and DT_m by 2-3°C and 8.5-12°C only, respectively, without any significant change in the integral curve profile correlates with early stages of inflammatory processes that corresponds to published data [19].

The comparison of plasma/serum melting curves (**Figure 5**) shows that the heat absorption deconvolution peak with T_m = 59 ± 1°C, DT = 2.5 ± 0.7°C is observed in case of BC stages II and IV, which is not seen in case of norm (**Figure 5**), even after deconvolution of over 154 healthy female subjects, 12 to 71 years old. In addition to this, it is observed a significant distribution of heat between the deconvolution peaks, and also some significant changes in T_m, DT_m, DH_m and DC^{max} (**Figure 5A**, **5B**) in comparison with healthy women. These changes may be connected with a strong interaction between plasma proteins. This conflicts with principles of independent melting of main proteins in healthy human blood plasma [18], which give us a reason to deconvolute DSC melting curves. Deconvolution of plasma curves revealed that the deconvoluted peak at about 67°C reflects melting of transferring and cellulo plasmin, the peak at 70°C corresponds to melting of Ig, the

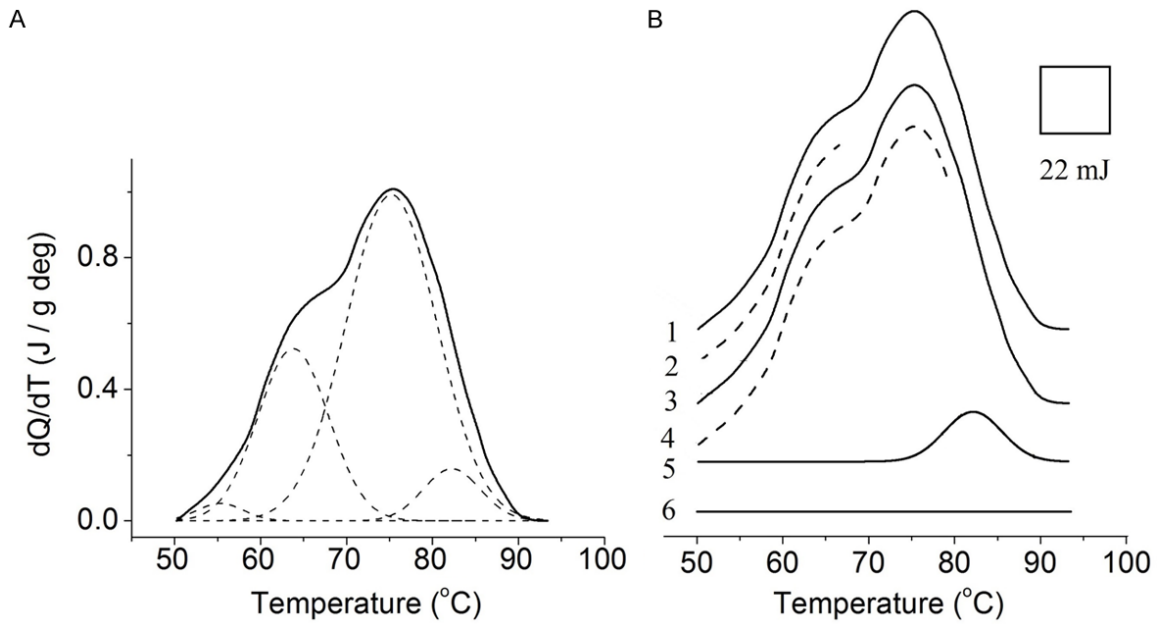

Figure 6. A. Deconvolution of Octagam® 5% sterilized solution made from human plasma. Total protein content of Octagam is at least 95% is human Immunoglobulin G: IgG1-60%, IgG2-32%, IgG3-7%, and IgG4-1%. Total enthalpy is 24.2 ± 2 J/g. B. Thermal treatment of Octagam® 5%. 1 - Native Octagam®. 2 - Heating to 67°C and then cooling to 25°C. 3 - Repeated scanning to 95°C. 4 - Heating again to 80°C and then cooling to 25°C. 5 - Repeated scanning to 95°C and then cooling. 6 - Final scanning to 95°C.

peak at 78°C corresponds to Ig partly and α_1-antitripsin, and the peak at 82°C corresponds to the fat fraction of albumin [7, 15, 18] and IgG [6]. The multi-stage melting of immunoglobulins is confirmed by the data presented in **Figure 6A**. The DSC study has demonstrated that the first two stages of melting of Octagam at ~55°C and ~64°C are fully reversible, and the melting stages at ~75°C and ~82°C are not reversible after thermal treatment (**Figure 6B**). The observed changes in T_m, DT_m, DH_m and DC^{max} (**Figure 5A**, **5B**) in comparison with healthy women may be explained by some strong interactions between main plasma proteins. However, other explanations are not excluded. It is known that human serum albumin consists of three domains, which melting independently from each other *in vitro* at 64°C, 68°C and 78°C in a wide temperature range [32]. In blood plasma, these three domains are combined and create 2 independent domains, which melt cooperatively in narrow temperature ranges with DT = 5° and DT = 8°, respectively. This fact demonstrates a strong interaction of domains. It is also known that the terminal N fragments of albumin and its central part are in a fatless condition in healthy human plasma. Hence, multiple binding centers of albumin can bind not only to metal ions, fat acids, hormones and different drugs, but they might also bind to some BC-specific biological oncomarkers. It is possible that those biomarkers are TNV and TNC [15, 30]. We suggest that binding of biomarkers to the fatless albumin fraction causes changes to its dynamic structure, which results in weakened interactions between albumin domains. It is not excluded that similar effect occurs in case of immunoglobulins that is expressed by increase in albumin T_m by 4°C, DT_m by ~300%, and decrease in dQ/dT by ~300%, and slight changes in g-globulins, in comparison with the norm (**Figure 5A**, **5B**, **5D**).

It should be noted that a weak clear peak is always seen at deconvolution of DSC curve of plasma/serum when the first symptom of relapse is revealed (**Figure 5C**). Taking into account the fact that the melting enthalpy (ΔH_m) of globular proteins, including multidomain proteins, equals ~6.0 cal/g in the temperature range 65-70°C [32], we can calculate concentration of proteins that melt in the temperature interval 56-63°C with maximum at T_m = 59 ±

1°C using heath value calculated from the area under this peak. Protein concentration is 650 ± 120 μg/ml in case of BC stage II-IV, it is 120 ± 50 μg/ml in case of BC risk, and it is 150 ± 60 μg /ml in case of BC relapse (**Figures 1**, **2**, dot line).

In conclusion, DSC gives us a possibility to register melting parameters T_m and DT_m, and we can calculate DH_m and DC^{max} values of main plasma proteins, as well as quantity of the suspected biomarkers by deconvolution of the DSC curves. Hence, on the basis of precise DSC measurements, it is quite possible to distinguish any slight differences between thermodynamic parameters of cancer and non-cancer diseases and norm. To reach this goal, the above mentioned approach is really helpful for investigation of significant experimental material and their computer-processed DSC curves, because it is less probable that all T_m, DT_m, DH_m and DC^{max} parameters of main proteins will simultaneously coincide in case of various diseases.

Taking into account the fact that the modern DSC enables us to detect some changes using as little as 125-50 μl of samples that are scanned at a high speed 1°C-10°C per min, this new diagnosis method will ensure reliable results in a very short time for confirmation of early diagnosis, as well as for detection of initial condition of BC relapse.

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Disclosure of conflict of interest

None.

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