Biochimica et Biophysica Acta 1820 (2012) 1879–1885

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

# Calorimetry-based profiling of blood plasma from colorectal cancer patients

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ARTICLE INFO

*Article history:*
Received 21 May 2012
Received in revised form 17 July 2012
Accepted 3 August 2012
Available online 10 August 2012

*Keywords:*
Differential scanning calorimetry
Colorectal cancer
Blood plasma
Thermal transition
Excess heat capacity
Transition temperature

ABSTRACT

*Background:* Differential scanning calorimetry (DSC), a highly sensitive technique for resolving thermally-induced protein folding/unfolding transitions, recently was recognized as a novel tool for disease diagnosis and monitoring. To further elaborate this approach we have applied DSC in a study of blood plasma from patients with colorectal cancer (CRC) at different stages of tumor development and localization.
*Methods:* Blood plasma from patients diagnosed with CRC was analyzed by DSC. The CRC thermograms were compared to those of healthy individuals and patients with gastric cancer, and non-cancerous soft tissue inflammation. The thermodynamic parameters: excess heat capacity and enthalpy of the transitions corresponding to the most abundant plasma proteins, as well as transition and first moment temperatures were determined from the calorimetric profiles.
*Results:* The calorimetric profiles of blood plasma from CRC patients are found to be distinct from those of healthy individuals and those of patients with soft tissue, non-cancerous inflammation. Generally the CRC thermograms exhibit reduced heat capacity of the major albumin/globulin-assigned thermal transitions, lower enthalpy and a featureless high temperature region compared to healthy individuals.
*Conclusions:* A classification of blood plasma proteome from patients with colorectal cancer (CRC1, CRC2 and CRC3 groups, and subgroups within each group $CRC1_{1-2}$, $CRC2_{1-2}$ and $CRC3_{1-2}$) is proposed based on the derived thermodynamic parameters.
*General significance:* The presented data demonstrate a proof of principle and confirm that the DSC technique has a potential to monitor changes in the CRC blood plasma proteome. This study is a further step toward the validation of calorimetry as a diagnostic tool.



## 1. Introduction

Differential scanning calorimetry (DSC) appeared as a novel tool in biomedicine in 2007 when Chaires and co-workers revealed its potential for disease diagnostics performed on blood plasma [1–3]. A typical DSC thermogram of blood plasma has been established for healthy individuals, whereas for diseased subjects (including oncopatients) it was shown to differ [1–6].

DSC is a highly sensitive technique that precisely measures the thermally-induced conformational transitions of biomolecules and allows the determination of the thermodynamic parameters of protein denaturation (folding/unfolding) [7]. The interest in applying DSC on blood plasma in cancer diagnostics is high since it is non-invasive for the patients and provides fast in situ monitoring of changes in the thermodynamic behavior of blood plasma/serum. Modifications of plasma DSC thermogram of a diseased subject are thought to be related either to alterations in plasma protein content or to shifts in their characteristic denaturation temperature due to ligand and/or tumor-specific secretory marker binding (interactomics). So far the DSC approach was applied to small cohorts of cancer patients [1–3,5,6]. Recently DSC investigation on multiple myeloma has been published by our research group [8]. These studies have identified specific disease-related calorimetric features however the validation of DSC as a useful tool for disease diagnostics and monitoring requires large-scale investigations of various diseases. As a step toward this goal, hereby, we report on an in-depth DSC study on a heterogeneous cohort of colorectal cancer (CRC) patients diagnosed with different tumor-node-metastasis (TNM) stages.

Although the DSC data reveal heterogeneity in the thermograms some common characteristics for the majority of the CRC plasma

*Abbreviations:* DSC, differential scanning calorimetry; CRC, colorectal cancer; GC, gastric cancer; STI, non-cancerous soft tissue inflammation; TNM, (tumor, lymph nodes, metastasis) stage; $T_{max}$, temperature maximum of the transitions; $T_{1M}$, temperature of the first moment; $c_p^{ex}$, excess heat capacity; $\Delta H_{cal}$, total enthalpy

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http://dx.doi.org/10.1016/j.bbagen.2012.08.001

**Table 1**
Clinical description of the CRC patients.

| Number of patients | 74 |
|---|---|
| Mean age (interval) | 63 ± 11 |
| Gender, M/F | M (39), F (35) |
| Stages | 55 cases |
| T2-T4N0M0 | 54.7% |
| T4-N1-2M0 | 18.9% |
| T4N0M1-M2 | 3.8% |
| T4N1-N2M1-M2 | 22.6% |
| After surgery | 19 cases |

profiles are to be noted: (i) strong variation in the albumin- and globulin-related thermal transitions and (ii) featureless high temperature region of the DSC thermograms compared to that of healthy individuals. Patients diagnosed with another epithelial cancer, namely gastric cancer (GC), show thermograms similar to certain CRC subtypes. In addition a pilot study on patients with soft tissue inflammation (STI) is also presented, which reveals features distinct from the ones found for CRC patients, GC patients and healthy individuals.

## 2. Material and methods

### 2.1. Individuals

The population under study included a control group of 32 healthy volunteers (of age 34–81 years, the mean age and the standard deviation being 57 ± 14), 74 patients with CRC (63 ± 11 years of age) (Table 1), 8 patients with GC (59 ± 15 years of age) and 6 with non-cancerous soft tissue inflammation. The patients selected for the study have not received chemo- and radiotherapy. Among the 74 CRC patients 55 varied in the disease stage determined by the TNM staging system: 54.7% of the CRC patients were without lymph node involvement and distal metastasis (T1-T4N0M0), 18.9% were lymph node positive and without distal metastasis (T4N1-2M0), 3.8% had distal metastasis but were lymph node negative (T4N0M1-2) and 22.6% were lymph node positive and had distal metastasis (T4N1-2M1-2), whereas the other 19 had undergone surgical intervention (Table 1).

### 2.2. Blood sample preparation

3 mL blood was centrifuged for 15 min at 900 RCF in Venosafe plastic tubes (Plasma gel). The supernatant (blood plasma) was carefully removed, diluted twice in PBS buffer, and used for subsequent DSC measurements.

### 2.3. Protein analysis

Protein concentration of the plasma samples is determined by the biuret method [9]. CEA, CA19-9 and CA 125 tests and staging determination were performed at the clinical laboratory of the National Oncology Hospital, Sofia, Bulgaria.

### 2.4. Differential scanning calorimetry

DSC thermograms were detected using DASM1 (Privalov, BioPribor)-build-in highly sensitive calorimeter and the data were analyzed with the Origin software package. The samples were heated at a scanning rate of 0.8 °C/min from 20 °C to 95 °C. The thermograms were normalized to the total protein content and corrected for the instrumental base line. The following parameters were determined: transition temperature, $T_{max}$ (temperature maximum of the successive transitions); temperature

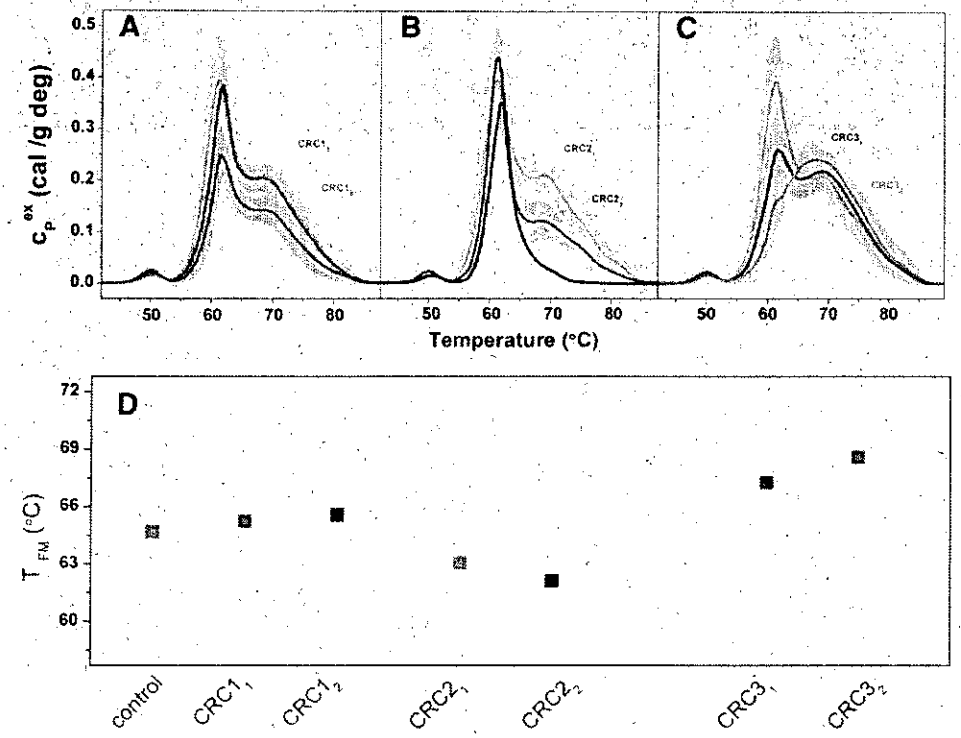


**Fig. 1.** Characteristic DSC profiles of blood plasma proteome from patients with CRC. Average DSC profiles (solid lines) and standard deviations (shading) are shown for the healthy individuals (cyan line/light gray shading, all panels) and CRC subgroups. Panel A: $CRC1_1$, red solid line and pink shading and $CRC1_2$, magenta solid line and blue shading; panel B: $CRC2_1$, olive solid line and green shading and $CRC2_2$, blue solid line and violet shading; panel C: $CRC3_1$, wine solid line and red shading and $CRC3_2$, orange solid line and dark yellow shading. For clarity the CRC groups are denoted in different colors in panels A–C. The first moment temperatures ($T_{FM}$) and their standard deviations for each study group are shown on panel D in the same color as the shading of the respective averaged thermograms in panel A–C.

**Table 2**
Thermodynamic parameters (average values and standard deviations) estimated from the DSC profiles and similarity parameters of healthy and diseased thermograms.

| Group | Figure | No. of cases | $c_P^{ex}{}_{T2}$ | $c_P^{ex}{}_{T2}/c_P^{ex}{}_{T3}$ | $c_P^{ex}{}_{T1}/c_P^{ex}{}_{T2}$ | $\Delta H_{cal}$ (cal/g deg) | $T_{FM}$ (°C) | P | r | ρ |
|---|---|---|---|---|---|---|---|---|---|---|
| Healthy[a] | | 32 | 0.42 ± 0.06 | 2.08 ± 0.29 | 0.04 ± 0.02 | 4.56 ± 0.73 | 64.7 ± 1.2 | – | – | – |
| Healthy[b] | | 8 | – | – | – | – | – | 0.797[1] (0.755*) | 0.988[1] (0.975*) | 0.850[1] (0.800*) |
| $CRC1_1$ | Fig. 1A | 16 | 0.40 ± 0.06 | 1.90 ± 0.23 | 0.06 ± 0.02 | 4.35 ± 0.88 | 65.3 ± 0.9 | 0.720 | 0.981 | 0.778 |
| $CRC1_2$ | Fig. 1A | 13 | 0.26 ± 0.04 | 1.92 ± 0.35 | 0.09 ± 0.03 | 2.90 ± 0.60 | 66.6 ± 1.1 | 0.578 | 0.971 | 0.658 |
| $CRC2_1$ | Fig. 1B | 5 | 0.44 ± 0.05 | 3.90 ± 1.64 | 0.14 ± 0.15 | 3.40 ± 0.43 | 63.1 ± 0.6 | 0.715 | 0.967 | 0.771 |
| $CRC2_2$ | Fig. 1B | 2 | 0.32 ± 0.13 | 12.10 ± 2.50 | 0.05 ± 0.01 | 1.90 ± 0.04 | 62.1 ± 0.3 | 0.370 | 0.854 | 0.456 |
| $CRC3_1$ | Fig. 1C | 27 | 0.31 ± 0.06 | 1.31 ± 0.14 | 0.11 ± 0.14 | 4.19 ± 0.99 | 67.3 ± 1.0 | 0.728 | 0.905 | 0.768 |
| $CRC3_2$ | Fig. 1C | 7 | 0.24 ± 0.02 | 0.69 ± 0.13 | 0.08 ± 0.01 | 4.04 ± 0.44 | 68.6 ± 0.7 | 0.630 | 0.700 | 0.647 |
| STI | Fig. 4 | 6** | 0.34 ± 0.09 | 1.33 ± 0.59 | 0.13 ± 0.01 | 4.52 ± 0.56 | 67.6 ± 1.3 | 0.706 | 0.874 | 0.745 |

Heat capacity at transition T2 ($c_P^{ex}{}_{T2}$); ratios between the heat capacities at T2 and T3 ($c_P^{ex}{}_{T2}/c_P^{ex}{}_{T3}$), and at T1 and T2 ($c_P^{ex}{}_{T1}/c_P^{ex}{}_{T2}$) transitions; total enthalpy ($\Delta H_{cal}$); first moment/temperature ($T_{FM}$); average values of spatial distance metric P, Pearson's correlation coefficient r and similarity metric parameter ρ.

[1] Values for the additional set of 8 healthy test thermograms (Healthy[b]) analyzed relative to the control reference set (Healthy[a]) of 32 DSC profiles.

* Values reported by Fish et al. [10] for 85 individual healthy thermograms compared with a reference set of 100 thermograms.

of the first moment, $T_{FM}$ (the temperature at the half calorimetric thermogram area, as defined in Garbett et al. [1]); excess heat capacity, $c_P^{ex}$, and enthalpy $\Delta H_{cal}$ (the integrated area under the heat capacity profile) of the thermal transitions.

The collected thermograms were also analyzed applying the statistical methodology developed by Fish et al. [10] for the classification of DSC curves relative to a comparative reference set. This analytical tool (described in detail in [10]) was used to determine similarities between a set of reference thermograms and a test (disease) thermogram by a similarity metric parameter ρ, that combines two factors: similarities in shape (Pearson's correlation coefficient, r) and in space (spatial distance metric P). The similarity parameters were determined for a number of test healthy thermograms (8) and for the CRC/STI subgroups (defined below) of thermograms related to a control reference set according to [10] in the temperature range 55–85 °C.

## 3. Results

### 3.1. Blood plasma DSC profiles of healthy individuals

DSC thermograms of 32 healthy individuals were recorded and a typical "healthy" DSC profile, very similar to the one presented by Garbett et al. for 100 individuals [3], was found (Fig. 1). It is characterized by four well resolved transitions at about 50 °C, 62 °C, 70 °C, 82 °C and a shoulder at ca. 76 °C. The successive transitions are denoted further on as T1 ($T_{max} \approx 50$ °C), T2 ($T_{max} \approx 62$ °C), T3 ($T_{max} \approx 70$ °C), T4 ($T_{max} \approx 76$ °C) and T5 ($T_{max} \approx 82$ °C) (Fig. 1). According to Garbett et al. [2] T1 transition can be attributed mainly to fibrinogen; T2 to albumin; T3 to immunoglobulins (Ig) and to albumin's minor, tail, transition; T4 to complement protein C3, IgA, IgG and albumin, while T5 has significant contribution from IgG and transferrin. Their respective $c_P^{ex}$ are denoted with subscripts T1–T5. One should keep in mind that other low abundant proteins contribute to those transitions as well though to a lesser extent because of their low plasma concentration level [2].

No significant difference was observed in the thermograms of males and females, with respect to their transition temperatures, as well as the enthalpy $\Delta H_{cal}$ (4.56 ± 0.73 cal/g deg). The heat capacity ratio $c_P^{ex}{}_{T2}/c_P^{ex}{}_{T3}$, i.e. the heat capacities corresponding mainly to albumin and globulins, has a value close to 2 for all samples (Table 2). The temperature of the first moment $T_{FM}$ is identical for all healthy individuals, ca. 65 °C, which is close to the value already published in [2].

### 3.2. Blood plasma DSC profiling of CRC patients

DSC analysis of plasma samples from CRC patients revealed that all thermograms differ from the typical "healthy" one and in 78% of the cohort this difference is very pronounced. In the remaining 22% the differences though significant are not so dramatic. Three main groups of CRC thermograms CRC1–3 are defined depending on the $c_P^{ex}{}_{T2}/c_P^{ex}{}_{T3}$ ratio which is: (i) slightly lower (Fig. 1A); (ii) significantly higher (Fig. 1B) and (iii) dramatically lower (Fig. 1C) than the one found for the healthy control (Table 2).

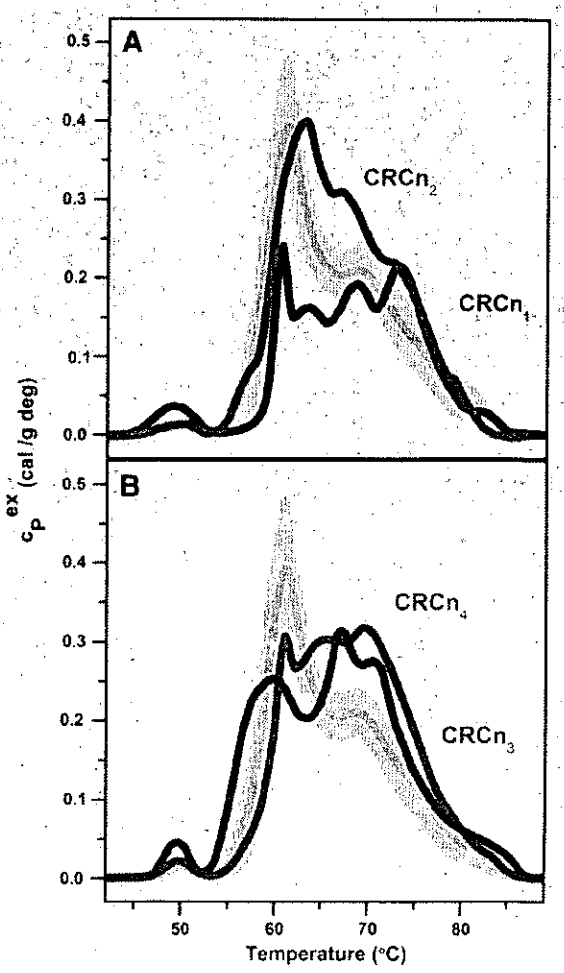


**Fig. 2.** DSC profiles of blood plasma from patients with CRC not included in the classification CRC1–3. The thermograms are designated as: $CRCn_1$ (navy), $CRCn_2$ (red) (A), $CRCn_3$ (purple) and $CRCn_4$ (green) (B). The cyan solid line and light gray shading represent the averaged "healthy" group.

Each of the defined CRC groups can be divided into two subgroups (denoted with subscripted numbers) which differ in the heat capacity values at transitions T2 and T3, and the total enthalpy $\Delta H_{cal}$. Among all groups the $CRC1_1$ subgroup is the closest to the "healthy" one, the ratio $c_P^{ex}{}_{T2}/c_P^{ex}{}_{T3}$ and $\Delta H_{cal}$ being almost indistinguishable from the "healthy" values, but the high-temperature shoulder/transitions are not well defined. For the $CRC1_2$ subgroup $\Delta H_{cal}$ and both $c_P^{ex}{}_{T2}$ and $c_P^{ex}{}_{T3}$ values are strongly reduced, but the $c_P^{ex}{}_{T2}/c_P^{ex}{}_{T3}$ ratio does not significantly differ from those of $CRC1_1$ and the control (Table 2).

The other two groups of thermograms, CRC2 and CRC3 show opposite tendencies in the alteration of the $c_P^{ex}{}_{T2}/c_P^{ex}{}_{T3}$ ratio, which is much higher for the former and much lower for the latter as compared to the control one, and $\Delta H_{cal}$ is reduced to a different extent (Table 2).

The $c_P^{ex}{}_{T2}/c_P^{ex}{}_{T3}$ value for the CRC2 thermograms increases dramatically (they vary in the range 2.26–14.6) due to the strongly reduced $cp^{ex}{}_{T3}$ value (Fig. 1, Table 2). Characteristic features of the CRC2 group of thermograms are the highly cooperative calorimetric profile, most remarkable for the $CRC2_2$ subgroup (Fig. 1B) and $T_{FM}$ strongly shifted to lower temperatures (Fig. 1D, Table 2).

In sharp contrast to CRC2, rather broad thermograms (Fig. 1C) and lower $c_P^{ex}{}_{T2}/c_P^{ex}{}_{T3}$ values are characteristic for $CRC3_1$ and $CRC3_2$ subgroups of thermograms, ca. 1.3 and 0.69 respectively, i.e. about one-half of the control (Table 2). The $CRC3_2$ subgroup exhibits a significant decrease in T2 transition whereas the heat capacity of T3 remains unchanged, thus the main transition corresponds to the third successive transition. The CRC3 group is also unique because of the significant shift in $T_{FM}$ to higher temperatures (from ~65 °C for the "healthy" group to 67.3 °C, for $CRC3_1$, and to 68.6 °C, for $CRC3_2$, Fig. 1D, Table 2).

A small cohort of the recorded CRC thermograms (only four of the analyzed CRC blood plasma samples, i.e. 5% of the presented CRC cases, regarded as outliers) shows features distinct from the majority of cases included in the $cp^{ex}{}_{T2}/cp^{ex}{}_{T3}$ ratio-based classification. These individual thermograms, $CRC_{n1-n4}$, are presented in Fig. 2. The $cp^{ex}{}_{T2}/cp^{ex}{}_{T3}$ values of those samples are in the range between 0.8 and 1.8. The four CRC cases (Fig. 2) exhibit a higher number of transitions/shoulders (5 or 6) and additional transitions appear at 63–67 °C, as compared to the control and the other CRC cases. The $cp^{ex}{}_{T1}$ increases in two of the cases while in three of them the 82 °C transition is preserved. Their $T_{FM}$ (66.3–68.5 °C) is close to the one found for the CRC3 group (67.3–68.5 °C), i.e. shifted to higher temperature compared to the control. The $CRC_{n1-n4}$ cases, all female individuals, are associated with a spread from the primary carcinoma to the epithelium of the neighboring tissues and adjacent organs, as reported in the clinical chart.

In addition to significant differences in $cp^{ex}{}_{T2}/cp^{ex}{}_{T3}$ ratios some common features can be identified for the majority of CRC DSC profiles (with the exception of those presented in Fig. 2): i) T1 is present in all CRC thermograms and its $T_{max}$ is not shifted (Table 2); ii) the $c_P^{ex}{}_{T1}/c_P^{ex}{}_{T2}$ ratio is more than double that of healthy controls (0.04) for CRC3 group and $CRC1_2$ and $CRC2_1$ subgroups (>0.09), while not significantly altered in the rest of the subgroups (for $CRC1_1$ and $CRC2_2$ it is 0.05–0.06) (Table 2); iii) no shift in the $T_{max}$ of T2 and T3 transitions is observed; iv) the T4 shoulder and the last transition, at ca. 82 °C, are significantly decreased or not resolved in CRC patients (Fig. 1). The lack of the latter transition, attributed to IgG and transferrin, might indicate either a strong reduction in their level in the plasma of CRC patients up to an almost undetectable amount, or else its transition is shifted, suggesting a significantly

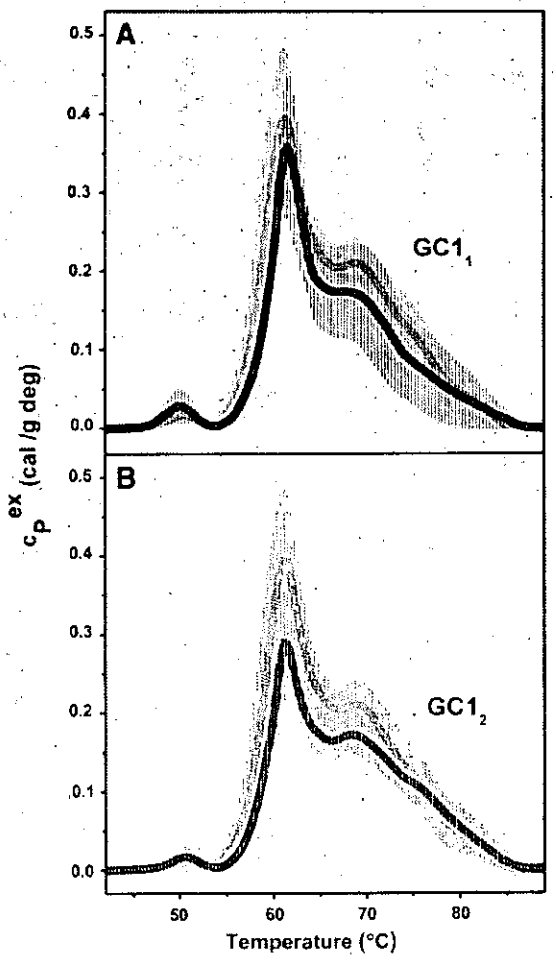


Fig. 3. Characteristic DSC profiles of gastric cancer. Average DSC profiles (solid lines) with standard deviations (shading) are presented for $GC1_1$ (dark blue line and cyan shading) (A) and $GC1_2$ (purple line and light magenta shading) (B). Also shown is the DSC profile for the healthy group (cyan line and light gray shading).

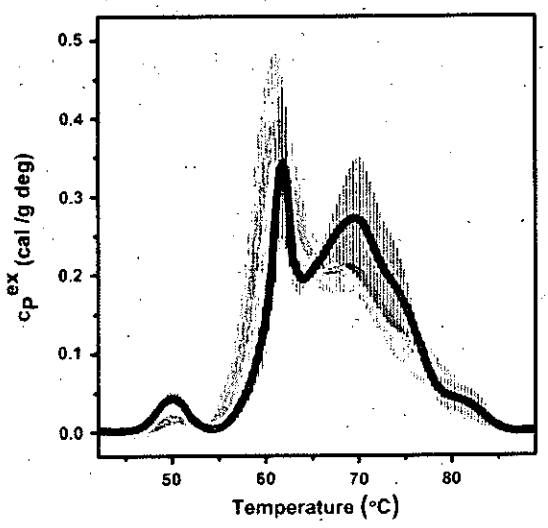


Fig. 4. Comparison of blood plasma from patients with non-cancerous soft tissue inflammation and the healthy thermogram. Average DSC thermogram for non-cancerous inflammation (black line) and that of healthy individuals (cyan line). The shading represents the standard deviation for each group (violet, inflammation; light gray, healthy group).

altered thermal stability of these blood plasma proteins; v) featureless high temperature (70–90 °C) region (Fig. 1) compared to that of healthy individuals.

The similarity metrics for the CRC subgroups in comparison to the control healthy reference set, estimated according to [10], vary in the following ranges $0.370<P(T)<0.728$; $0.700<r<0.981$; $0.456<\rho<0.778$ (Table 2). These values are significantly lower, especially for the subgroups denoted by $CRC1_2$, from those estimated for the test healthy thermograms, not included in the reference set (Table 2). The values of the metrics P and r for a small additional group of 8 healthy test thermograms only slightly differ from those published in [10] due to the much smaller set of healthy test thermograms used in our work (Table 2). This analysis demonstrates that the CRC classification based on the thermodynamic parameters $c_p^{ex}{}_{T2}/c_p^{ex}{}_{T3}$, $\Delta H_{cal}$ and $T_{FM}$ conforms to the applied statistical tool.

### 3.3. DSC profiles of patients with gastric cancer

The thermograms of blood plasma from patients with gastric cancer (8 cases) are presented in Fig. 3. Likewise the CRC classification, considering the $cp^{ex}{}_{T2}/cp^{ex}{}_{T3}$ ratios, two subgroups (again denoted with subscripted numbers) can be distinguished, $GC1_1$ and $GC1_2$, the $cp^{ex}{}_{T2}/cp^{ex}{}_{T3}$ ratio being significantly lower for the $GC1_2$ ($2.19\pm0.25$) than for $GC1_2$ ($1.39\pm0.14$) subgroup. It can be seen that these subgroups resemble very much the $CRC1_1$ and $CRC3_1$ (compare Figs. 1A and C, and 3). Further study on a larger cohort of patients with GC would prove similarities and/or differences with the CRC classification.

### 3.4. Plasma DSC profiles of patients with soft tissue inflammation

Importantly, the thermograms of plasma from patients with soft tissue, non-cancerous, inflammation (Fig. 4) present the same number of successive transitions as the control one and no substantial shift in the respective $T_{max}$s but a significantly increased $T_{FM}$ (Table 2) was observed. The excess heat capacities, however, vary significantly. The $c_p^{ex}{}_{T1}$ (fibrinogen), $c_p^{ex}{}_{T3}$ and $c_p^{ex}{}_{T4}$ (globulins) significantly increase, whereas that of albumin, $c_p^{ex}{}_{T2}$, decreases. Importantly, the 82 °C transition is preserved in those patients. Therefore, the thermodynamic behavior is not comparable to that of cancer, colorectal and gastric, plasma proteome. The similarity metrics also prove that the STI thermograms are very distinct from the healthy ones (Table 2). These data are in line with the findings that albumin level decreases, whereas fibrinogen increases upon inflammation [11,12].

## 4. Discussion

Colorectal and gastrointestinal cancers remain among the most frequent malignant diseases worldwide with poor prognosis and low percent of survival, particularly when detected in an advanced stage. Besides the manifold approaches for identification of CRC biomarkers no significant progress in CRC treatment has been achieved so far. The same holds true for another problematic issue which is an important part of CRC management, namely the high percentage of CRC patients developing recurrent disease or metastasis following surgery, or showing resistance to therapy.

Although CRC appears phenotypically homogeneous, it has been shown to be heterogeneous on a molecular level. Multiple CRC molecular subtypes based on microsatellite and chromosomal instability, presumably with prognostic and predictive significance, have been found (as discussed in [13–17]). A systems biology approach (based on Boolean logic) that integrates the available genetic, transcriptomic, epigenetic and molecular information on cancer biology has been applied to CRC and has identified novel CRC associated genes and oncogenic transcription factors [18].

In this work we present a different, thermodynamic viewpoint on the heterogeneous CRC. Our data reveal several CRC groups with different thermodynamic features which are suggested to originate from modification of plasma proteome and might help the current understanding of CRC in several directions discussed below.

### 4.1. Contribution of microcalorimetry to CRC research

This work presents a thermodynamic investigation of blood plasma proteome from patients with CRC and a comparison with the one from "healthy" individuals, patients with gastric cancer and soft tissue inflammation. Although the vast majority of the cancer patients are in an advanced stage of the disease, the thermograms do not show one characteristic CRC calorimetric profile, but can be divided into three main groups (six subgroups in total) depending on their respective thermodynamic parameters. Heterogeneity most probably stems from the complex character of the disease, which on a molecular level is expressed in strongly modified molecular interactions.

All six CRC subgroups, particularly $CRC1_2$ and $CRC2_2$, show reduced enthalpy change $\Delta H_{cal}$ as compared to the healthy thermogram (Table 2). The enthalpy reduction might reflect destabilization of some plasma proteins, probably globulins, considering that the value of the calorimetric enthalpy of protein unfolding correlates with the protein stability and/or decrease in the concentration of some plasma proteins. On the contrary, the enthalpy change has a slightly higher value for three of the CRC outliers, $CRC_{n2}$–$CRC_{n4}$ ($\Delta H_{cal}$ ~5.5 cal/g deg), not included in the calorimetric classification, that should reflect stabilization of certain plasma proteins, which unfold with midpoint transition temperatures $T2_1$ and $T2_2$, albumin being one of the possible candidates. Though 25% of the CRC patients have undergone surgical intervention 1 to 3 months before collection of the plasma samples, their thermograms do not coincide with the "healthy" profile but rather belong to some of the CRC calorimetric profiles. This finding demonstrates that the thermodynamic features of the blood plasma remain affected even after surgery, reflecting long-term modifications of the plasma proteome.

Furthermore, it appears that blood plasma from patients with a common TNM stage shows distinct DSC profiles. For example 54.7% of the CRC cases have T1-T4N0M0 stages, i.e. without metastasis and without lymph node involvement, however their thermograms are spread between all CRC groups with the exception of $CRC2_2$. It is to be noted, however, that the TNM staging does not always correlate with the disease progression. Likewise, elevated levels of the CRC markers CEA, CA19-9 and CA125 are found in less than a half of the cohort under study, which agrees with the fact that increased levels of CEA marker is not always detected [19]. The statistical evaluation of the CRC subgroups of thermograms proves their strong difference from the healthy controls. Thus the algorithm for quantitative classification of DSC profiles, demonstrated for the case of systemic lupus erythematosus [10], holds for CRC cases as well.

### 4.2. Calorimetric discrimination of malignancies

We have recently reported distinct thermodynamic profiles for blood serum proteome of patients diagnosed with multiple myeloma (MM), another largely heterogeneous malignancy [8]. The correlation of the DSC profiles with serum protein electrophoresis data revealed that the observed changes in the thermodynamic features of the most abundant blood serum proteins (albumin and immunoglobulins) could be assigned not only to changes in their concentration but rather to severe changes in their conformation and thermal stability, ascribed to protein–protein and/or ligand interactions. At this point it is clear that the featureless high-temperature region and the disappearance of the IgG/transferrin-assigned transition (occurring at 82 °C in blood plasma and at 85 °C in blood sera) are common for CRC, GC and the majority of the MM cases [8]. However, a shoulder at this transition is observed in amyotrophic lateral sclerosis,

ovarian cancer and melanoma, where it is more pronounced than in the control [3]. So far, only for endometrial and cervical cancers this transition was found to coincide with the one found in the healthy set [3]. These data suggest that changes in the $T_{max}$ and $c_p^*$ of T5 transition might serve as malignancy indicators, at least for certain cancers.

Most of the studied CRC cases, similar to MM, are characterized by modified ratios between the excess heat capacities of albumin and globulins, however in contrast to MM, no apparent shift in their respective $T_{max}$ is observed for the CRC cases. Conversely, two limiting CRC cases are to be noted, namely the $CRC2_2$ and $CRC3_2$ subgroups (Fig. 1B and C). The $CRC2_2$ thermograms exhibit highly cooperative thermograms where all transitions merge into one. Whereas in $CRC2_2$ the globulin's transition is hardly observed, in $CRC3_2$ the albumin one is dramatically decreased and appears only as a shoulder of the main broad, globulin-assigned transition. Although at this point we have no exact data for the proteome composition of these cases, it cannot be assumed that the level of albumin or globulins is reduced to such a great extent. It seems more appropriate to suggest that their thermal stability is strongly affected, thus their thermal transitions overlap and/or they unfold together as macromolecular complexes. Even though these two groups represent only a small part of the cohort (12%) further investigation of their proteome composition might provide important information for the cancer-related interactomics. Although in 22% of CRC (this study) and 10% of MM [8] cases the thermograms do not differ significantly from the control set, the remaining CRC and MM subsets (78% and 90%, respectively) generally differ from each other, which strengthens the DSC potential for disease discrimination. The validation of DSC as a diagnostic tool requires high specificity, i.e. ability to distinguish between different diseases as well as between neoplastic disorders with different origins and localizations. Indeed, when comparing myeloma and CRC, as discussed above, DSC profiles strongly differ. On the other hand comparison of CRC and GC, both of them originating from epithelial dysplasia, but differing in their localization, reveals common characteristics. Thus, it appears that the DSC technique has a strong potential to discriminate malignancies with respect to their nature and localization which however, requires further systematic studies. DSC, once validated, might be useful as an additional test for CRC detection complementing the two currently applied worldwide strategies for early CRC diagnosis (fecal occult blood test and colonoscopy).

### 4.3. Thermodynamic discrimination between cancer and inflammation

The relation between inflammation and cancer is a long-standing issue, recently recognized as the seventh hallmark of cancer [20,21]. The inflammation (chronic and persistent) is a risk factor for cancer development, since the inflammatory component is shown to build up the microenvironment of most neoplastic tissues [20–22]. Thus it is of interest to establish whether the characteristic cancer thermodynamic features correlate with inflammatory processes. Indeed our data on patients with STI, but not cancer, reveal a characteristic DSC profile that reflects reduced albumin and increased fibrinogen and immunoglobulin levels, which is different from the profiles found for patients with cancer. Although this remains to be validated with a larger group of such patients, it certainly brings out the possibility to monitor the inflammatory process by DSC. It should also be noted that the four cases of CRC, not included in the DSC-based classification (presented in Fig. 2) and which are associated with the spread of the tumor toward adjacent tissues, exhibit some thermodynamic features common with those of STI, i.e. increased T1, sharp T2, well defined T4 and preserved T5 transitions.

In summary, this work reveals colorectal cancer-specific alterations in the thermodynamic behavior of blood plasma proteome. Three CRC groups of thermograms are defined, based on the derived thermodynamic parameters. The presented findings contribute to the validation of calorimetry as a non-invasive tool for CRC diagnostics and discrimination of malignancies. Future investigations will extend this study in order to obtain higher statistics and better understanding of the observed changes in the thermodynamic properties of blood plasma and serum from cancer patients.

## Ethics statement

The analyzed blood samples were provided by the National Oncology Hospital, Sofia, Bulgaria after obtaining written informed consent from the patients. This study was specifically approved by the ethics committee of the National Oncology Hospital.

## Acknowledgements

We thank Prof. F. Goñi for the critical reading of the manuscript, and Dr. Churishki and C. El-Tal from the clinical laboratory of the National Oncology Hospital for their contribution in supplying blood plasma samples. This work is supported by project no. BG051PO001-3.3-05/0001, grant scheme Science-Business, funded by Human Resources Development Operational Programme. S. K. is thankful to the World Federation of Scientists, Switzerland for the fellowship granted. S.G.T. was a visiting senior researcher, IKERBASQUE, Basque Foundation for Science.

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