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Thermal Liquid Biopsy for monitoring melanoma patients under surveillance during treatment: A pilot study

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Abstract

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CONFLICT OF INTEREST STATEMENT

NCG is a co-inventor on a patent application describing approaches for the analysis of DSC plasma thermogram data and their use for diagnostic classification (Garbett, N.C., and Brock, G.N. "Methods of Characterizing and/or Predicting Risk Associated with a Biological Sample Using Thermal Stability Profiles," U.S. PCT Application PCT/US16/57416, Oct. 2016). NCG is a consultant for the calorimetry instrument supplier TA Instruments, Inc. involved in education for microcalorimetry applications and characterization of microcalorimetry instrument performance. This does not alter the authors' adherence to all policies on sharing data and materials.

Background: Differential Scanning Calorimetry (DSC) is a technique traditionally used to study thermally induced macromolecular transitions, and it has recently been proposed as a novel approach for diagnosis and monitoring of several diseases. We report a pilot study applying Thermal Liquid Biopsy (TLB, DSC thermograms of plasma samples) as a new clinical approach for diagnostic assessment of melanoma patients.

Methods: Multiparametric analysis of DSC thermograms of patient plasma samples collected during treatment and surveillance (63 samples from 10 patients) were compared with clinical and diagnostic imaging assessment to determine the utility of thermograms for diagnostic assessment in melanoma. Nine of the ten patients were stage 2 or 3 melanoma subjects receiving adjuvant therapy after surgical resection of their melanomas. The other patient had unresectable stage 4 melanoma and was treated with immunotherapy. Two reference groups were used: (A) 36 healthy subjects and (B) 13 samples from 8 melanoma patients who had completed successful surgical management of their disease and were determined by continued clinical assessment to have no evidence of disease.

Results: Plasma thermogram analysis applied to melanoma patients generally agrees with clinical evaluation determined by physical assessment or diagnostic imaging (~80% agreement). No false negatives were obtained from DSC thermograms. Importantly, this methodology was able to detect changes in disease status before it was identified clinically.

Conclusions: Thermal Liquid Biopsy could be used in combination with current clinical assessment for the earlier detection of melanoma recurrence and metastasis.

General Significance: TLB offers advantages over current diagnostic techniques (PET/CT imaging), limited in frequency by radiation burden and expense, in providing a minimally-invasive, low-risk, low-cost clinical test for more frequent personalized patient monitoring to assess recurrence and facilitate clinical decision-making.

Graphical abstract

Keywords

Melanoma; Differential scanning calorimetry (DSC); Non-invasive test; Early diagnostic and prognostic; Patient surveillance

1. Introduction

Melanoma is the least common but deadliest skin cancer. In the US, it accounts for less than 5% of all skin cancer cases [1], but it represents the majority of skin cancer deaths [2]. For 2017, more than 87,000 new cases of melanoma are estimated in the US, making it the fifth

most common cancer in men and the sixth most common in women of all age groups [3]. While the average age of diagnosis is 64, melanoma is the third most common cancer among 20–39 year old women, the second most common cancer in 20–39 year old men and 25% of patients are diagnosed at less than the age of 45 [3]. According to the World Health Organization, about 232,000 new cases of melanoma are diagnosed worldwide each year.

Melanoma cases in the US have experienced a dramatic increase over the past few decades. The incidence has increased 15 times in the last 40 years, an increment larger than any other type of cancer [4]. In the UK, a similar incidence rise has been observed [5]. The mortality rate for melanoma (number of deaths per 100,000 people each year) has increased at a much slower pace and has remained stable over the past 10 years. This has been reflected in five year survival rates higher than 90% [6], which suggests patients are living longer after being diagnosed of a disease that traditionally exhibited a high mortality rate [7]. This may be due to earlier diagnosis, when tumors are still at a thinner depth, as well as improved treatment and surgical/therapeutic techniques [8].

Recurrence in melanoma patients is heavily dependent on tumor size and distant metastasis. Recurrence appears in 50% of patients with tumor thickness larger than 4 mm, increasing to 96% in case of distant metastasis [9]. This may occur at the level of regional lymph nodes (50%), local recurrences (20%), and distant sites (30%) [10]. Recurrence is most common during the first 2–3 years after treatment, but there are some cases of diagnosed patients with early-stage melanoma with recurrences 10 years after initial diagnosis [11]. Some studies concluded that routine computed tomography (CT) is the best way to detect asymptomatic recurrences within 3 years from the original melanoma diagnosis [12].

In melanoma, surveillance is complex due to the need to balance the cost-effectiveness and risks of routine surveillance imaging with a patient's desires. Several authors concluded that for stage I or II patients routine imaging is not cost-effective [13–15]. Moreover, imaging studies are not without risk to the patient, and unnecessary radiation exposure must be considered when a surveillance protocol is defined. Despite this fact, it has been reported that physicians prescribe diagnostic imaging tests to mitigate patient anxiety and to improve survivor quality of life [16].

Consequently, a complementary surveillance method that is simple, minimally-invasive, and low-risk could be a very convenient and useful approach for helping to reduce the adverse effects of imaging procedures. This could serve as a complementary diagnostic test where the minimally-invasive test could be applied for frequent patient assessment allowing an increased time interval between imaging procedures.

In the last ten years, differential scanning calorimetry (DSC) analysis of blood plasma has been revealed as a potential approach for the discrimination between healthy and cancer patients [17–20]. A DSC thermogram reflects the global denaturation profile for all blood plasma proteins present in the sample and the potential interactions between blood plasma proteins and metabolites. As a result of tumor development, the blood serum composition may be altered, because of the up- or down-regulation of proteins, or the presence of disease-related metabolites, and the plasma thermogram would reflect the distorted

denaturation profile of the blood proteins. Thus, the thermogram from a diseased subject can be quite different when compared to the thermograms from healthy subjects, and this technique could be employed as a clinical test for diagnosing, classifying, and monitoring patients [21]. In a previous study in our group, we demonstrated that DSC could be applied for classifying and staging gastric adenocarcinoma (GAC) patients. In addition, we offered new graphical tools and value ranges for the parameters differentiating among patients groups in order to discriminate healthy from diseased subjects with increased disease burden [22].

In this manuscript, we applied our methodology to characterize three subject groups: (A) 36 healthy controls, (B) 8 melanoma patients that had completed surgical management of their disease and were determined by continued clinical assessment to have no evidence of disease, and (C) 10 melanoma patients that were receiving or had recently completed treatment and were under surveillance for disease response or recurrence. We defined reference regions for subjects with no evidence of disease (healthy controls and melanoma patients in remission) and compared these to longitudinal thermograms for the 10 melanoma patients under surveillance. We correlated clinical assessment from physical exams, surgeries and imaging procedures with the DSC thermogram results. Within our 3rd subject group, the 10 patients were further split into three groups. Group A comprised five patients with no evidence of disease throughout their surveillance period with agreement between clinical assessment and DSC thermograms. Group B comprised three patients with active disease or disease recurrence during their surveillance period with concordant results from both clinical and DSC assessment. Group C comprised two patients with no evidence of disease during their surveillance with discordant results between clinical assessment and DSC thermograms.

We show that the DSC thermogram method demonstrates preliminary utility for detecting metabolic changes in oncology patients under surveillance (e.g. melanoma remission or recurrence). Moreover, our results suggest that thermograms could be particularly useful in detecting changes in disease recurrence cases where imaging techniques usually do not provide positive results (e.g. a tumor cannot be detected due to its small size or imaging is limited in its interval of use for avoiding risk to the patient).

2. Material and methods

2.1. Plasma samples

The study protocol and patient consent procedures were approved by the University of Louisville Institutional Review Board (IRB# 08.0388, 10.0144, 14.0517). Melanoma patients attending the Multidisciplinary Melanoma Clinic at the James Graham Brown Cancer Center for initial evaluation, treatment, follow-up and surveillance were eligible and gave written informed consent for their blood plasma to be entered into a bio repository (IRB# 08.0388) and utilized for research purposes. The IRB specifically approved the use of plasma specimens from the bio repository for use in this study without the need for further consent (IRB# 10.0144, 14.0517). All specimens collected for the study were deidentified. Associated demographic and clinical information was collected by clinical trials office personnel and securely stored on the bio repository computer. The bio repository was

approved by the University of Louisville Institutional Review Board (IRB# 08.0388) and was fully HIPAA compliant. Specimens provided for DSC studies were coded by bio repository collection number. In this form, specimens were deidentified and blinded for demographic and pathologic disease status for unbiased data collection. Demographic and clinical status was subsequently provided for data analysis.

Blood was drawn into 5 mL green top (plasma; sodium heparin anticoagulant) vacutainers. Tubes were gently mixed by inversion 8–10 times immediately after blood collection to evenly distribute the anticoagulant additive followed by centrifugation at 3200 rpm for 10 minutes (BD-Clay Adams Compact II centrifuge). Separated plasma was carefully aspirated to avoid hemolysis or contamination of the separated blood phases, aliquoted and immediately stored at -80°C until analysis. All handling of specimens and specimen waste was in accordance with OSHA bloodborne pathogen procedures.

Two control groups were considered: healthy individuals ($n = 36$), and patients who had completed successful clinical management of melanoma and were shown by continued clinical assessment to maintain no evidence of disease ($n = 8$). Healthy individuals were archived data previously collected using commercial plasma samples and tested on an Automated Capillary DSC (MicroCal - Malvern Instruments, UK) and were consistent with data obtained on a Nano DSC Autosampler System (TA instruments, New Castle, DE). Melanoma controls who had completed successful surgical management of melanoma with continued clinical assessment showing no evidence of disease. The study group of melanoma patients under surveillance (MUSP) consisted of 10 melanoma patients, as a pilot study, for which imaging or clinical evaluation and DSC thermograms were available at the same time points (Table 1). Nine of the ten patients were stage 2 or 3 melanoma patients that attended the melanoma clinic over a period of one to two years for adjuvant therapy and surveillance after surgical resection of their melanomas. The other patient had unresectable stage 4 melanoma and was treated with immunotherapy. More details can be found in the supplementary data (Table S1).

2.2. Collection of DSC thermograms

Plasma samples (200 μL) were dialyzed against a standard phosphate buffer (1.7 mM KH_2PO_4 , 8.3 mM K_2HPO_4 , 150 mM NaCl, 15 mM sodium citrate, pH 7.5) for 24 hours at 4°C in order to achieve normalization of buffer conditions for all samples. To effectively dialyze such small volumes of plasma, we used Slide-A-Lyzer MINI dialysis devices (MWCO 3,500, 0.1 mL; Pierce, Rockford, IL) with each plasma sample (200 μL) split between two dialysis units to provide a sufficient volume to complete DSC analysis. Dialysis units were loaded with 100 μL of dialysis buffer and equilibrated overnight at 4°C against 1 L of dialysis buffer. Frozen plasma samples were thawed overnight at 4°C the evening before dialysis. On the morning of dialysis, the dialysis units were removed from the beaker, emptied of buffer and loaded with plasma samples. Samples were dialyzed against 1 L of phosphate buffer with buffer changes after three hours of dialysis, then after two periods of four hours with a final overnight dialysis period. Based on cost and reliability we routinely re-assembled washed dialysis units replacing the original dialysis membrane with cut-to-size Snakeskin Pleated Dialysis Tubing (Pierce, Rockford, IL). After dialysis, samples were

recovered from dialysis and filtered to remove particulates using Spin-X centrifuge tube filters (0.45 μm cellulose acetate; Corning Incorporated, Corning, NY). The final dialysis buffer was also filtered (0.2 μm polyethersulfone; Pall Corporation, Ann Arbor, MI) and used for all sample dilutions and as a reference solution for DSC studies.

DSC data were collected with a Nano DSC Autosampler System (TA instruments, New Castle, DE) which was serviced according to the manufacturer's procedures. Instrument performance was assessed using the biological standard lysozyme and was within manufacturer's specifications. Dialyzed samples were diluted 25-fold to obtain a suitable protein concentration for DSC analysis. Samples and dialysate, to load the instrument sample and reference chambers, respectively, were loaded into 96 well plates thermostated at 4°C within the instrument autosampler until DSC analysis. Sample volumes of 950 μL were required to provide sufficient volume to ensure proper rinsing and filling of the 300 μL thermal sensing area. Thermograms were recorded from 20°C to 110°C at a scan rate of 1°C/min with a prescan equilibration time of 900 seconds, although data analysis was performed only in the 40–95°C temperature range (see below). The instrument was cycled overnight by running multiple water-water scans (during the overnight dialysis period) followed the next morning by at least three buffer-buffer scans to condition the instrument chambers before running the sample set. In designing our experimental approach for the analysis of blood plasma samples we have carefully examined each aspect of the process: blood sample collection and handling; sample preparation for DSC analysis; instrument settings and analysis replicates; data analysis and interpretation. This methodological study has recently been published [23], and it was shown that sample long-term storage at –20 or –80°C and/or dialysis did not influence the DSC results compared with fresh plasma. Importantly, we demonstrated that plasma thermograms were robust to all analytical and pre-analytical variables examined. These studies enabled us to adopt a standard protocol for the analysis of clinical samples. Our standard protocol, based on the limited availability of sample aliquots and to provide reasonable analysis throughput, involved the collection of duplicate scans for each sample and batching of samples to ensure that DSC analysis is completed within a seven day window after initial thawing of each sample batch. For each sample set, we examined buffer scans collected at the beginning and end of a sample set and after single or consecutive samples scans to determine acceptable reproducibility and effective cleaning of the instrument chambers. We also compared sample scans collected after a buffer or sample scan and found it is possible to collect consecutive sample scans after extensive rinsing of the instrument chambers with little effect on the thermogram profile. We routinely compared duplicate sample scans collected under different run sequences and at different time points to ensure the thermogram profile was reproducible.

For this study, scans were performed in duplicate and raw DSC data were corrected for the instrumental baseline by subtraction of a suitable buffer reference scan followed by the multiparametric analysis described in section 2.3.

2.3. Data analysis

The direct measurement in the scanning calorimeter provides the thermogram: heat capacity at constant pressure of the sample as a function of the temperature, $C_p(T)$. We have

employed a previously developed phenomenological model, in which the thermogram is deconvoluted into several individual transitions, modeling each individual transition by the logistic peak or Hubbert function. As it was previously reported, the Hubbert function is able to reproduce (even better than a Gaussian function) the thermogram for the unfolding of a protein [22].

In the case of thermograms from melanoma patients, a minimum set of four component curves ($i = 1, \dots, 4$) was required to represent the thermograms (Figure S1):

$$C_P(T) = C_{P,0} + \sum_{i=1}^{N=4} \frac{4A_i \exp\left(-\frac{T-T_{c,i}}{w_i}\right)}{\left(1 + \exp\left(-\frac{T-T_{c,i}}{w_i}\right)\right)^2} \quad (1)$$

where A is the height of the peak, T_c is the center of the peak, and w is the width of the peak ($C_P(T_c \pm w) \sim 0.8 A$ and $C_P(T_c \pm 2w) \sim 0.4 A$). The offset parameter $C_{P,0}$ (found to be always very close to 0 in the data analysis) was included as an adjustable parameter to offset potential errors from baseline correction (in fact, the data analysis showed little sensitivity to the baseline correction procedure). Non-linear iterative regression analysis was performed using user-defined fitting routines in Origin 7 (OriginLab).

Adding more parameters (i.e., more components in the previous equation) did not improve the analysis; on the contrary, the function becomes over-parameterized with degeneracy and cross-correlation among the parameters occurring during non-linear fitting analysis. Therefore, for any given melanoma plasma thermogram, twelve parameters (A_i , $T_{c,i}$ and w_i , for each of the four components) were obtained as those providing the non-linear regression best fit to the experimental data. This set of parameters constituted the basis for the multiparametric comparative quantitative analysis aimed at establishing classification criteria among healthy subjects and melanoma patients. The subscript for each parameter indicates which component curve it is associated to, and those components are in turn ordered along the temperature axis (i.e., $T_{c,i}$'s are monotonically increasing).

Other useful statistical parameters were defined for each thermogram, as shown in the Results section. In particular, the area under the curve (AUC), and the average temperature or the first moment of the thermogram, T_{ave} , defined according to the following expressions:

$$AUC = \sum_j C_P(T_j) \Delta T_j \quad (2)$$

$$T_{ave} = \frac{\sum_j C_P(T_j) T_j}{\sum_j C_P(T_j)}$$

where j spans the entire range of temperatures (experimental points) in the thermogram in the analysis range (40–95°C).

The height of the second peak, A_2 , was identified as the transition with higher susceptibility to alterations in the plasma metabolic state as a result of the disease, and was selected as a normalizing factor (Figure S1 and S2). In addition to the amplification effect on the observed differences between thermograms, normalizing using A_2 makes protein concentration determination and normalization by concentration unnecessary during the data analysis. Thus, the normalized area under the curve, AUC_n , and the normalized heights, $A_{i,n}$, were defined as:

$$AUC_n = \sum_j \frac{C_p(T_j)}{A_2} \Delta T_j \quad (3)$$

$$A_{i,n} = \frac{A_i}{A_2}$$

It is important to indicate that only the parameter A_i is dependent on protein concentration (i.e., it would be affected by protein concentration normalization uncertainties), while $T_{c,i}$ and w_i are independent of normalizing factors (i.e., they are concentration- or scale-independent parameters). In addition, a polygonal plot was constructed with the normalized heights $A_{i,n}$, where each $A_{i,n}$ is taken as the distance from the center of a circle at four equally spaced directions in the plane of the circle, and the four points are linked forming an irregular polygon [22] (see Figure S3). The area of this normalized heights polygon, AP_n , was calculated as [22]:

$$AP_n = \sum_{i=1}^{N=4} \frac{\sqrt{3}}{4} A_{i,n} A_{i+1,n} \quad (4)$$

2.4. Data representation

Data from the multiparametric analysis (AUC_n , AP_n , and T_{ave}) were represented using either X, X/Y or X/Y/Z graphs (that is, mono-, bi- or tridimensional graphs), where X, Y and Z may be any of these parameters. Each control subject and each patient time point (e.g. initial diagnosis, after-treatment) was represented by a point in a tridimensional plot with AUC_n , AP_n , and T_{ave} as Cartesian coordinates. Healthy subjects considered as controls delimit the region corresponding to the absence of disease, which can be represented by an ellipsoid with a centroid located at the mean of each of these parameters ($\overline{AUC_n}$, $\overline{AP_n}$, $\overline{T_{ave}}$), with the standard deviation of each parameter (σ_{AUC_n} , σ_{AP_n} , and $\sigma_{T_{ave}}$) defining the boundaries of the ellipsoid. The location of each patient at a certain time point relative to the set of controls, inside or outside the control or “absence-of-disease” ellipsoid, provides information on the health status of that patient at that time. This can be quantified by calculating the Euclidean distance of each patient time point to the center of the control ellipsoid, the d-value, taking as a reference the average values of the parameters for the no evidence of disease (NED) control group:

$$d = \sqrt{\sum_{i=1}^3 \left(\frac{X_i - \bar{X}}{\bar{X}} \right)^2} = \sqrt{\left(\frac{AUC_n - \overline{AUC_n}}{\overline{AUC_n}} \right)^2 + \left(\frac{T_{ave} - \overline{T_{ave}}}{\overline{T_{ave}}} \right)^2 + \left(\frac{AP_n - \overline{AP_n}}{\overline{AP_n}} \right)^2} \quad (5)$$

where X_i are the different values of the three parameters ($AUC_n/AP_n/T_{ave}$) for a given patient at a certain time. To avoid any bias towards any given parameter, the d-value was calculated by normalizing each of the three parameters by each mean, respectively.

3. Results

3.1. Deconvolution of plasma thermograms

We have previously described [22] a multiparametric analysis approach applying logistic peak fitting to serum thermograms obtained from healthy subjects and gastric cancer patients for the classification of patients. Sample handling was very simple, involving the direct measurement of serum samples after a 1:25 dilution in phosphate buffered saline at pH 7.5. In the present work, sample handling was slightly different employing a dialysis procedure followed by 25-fold dilution. Thermograms from the present study required four component curves to reproduce the global thermogram. Figure S1 shows an example of differences between thermogram shapes from a healthy subject and a melanoma patient (corresponding to Sample 1 of Patient 8 of this manuscript). The individual deconvoluted transitions reflect major changes between the healthy and melanoma groups where changes in height, center and width of each transition were observed.

Deconvolution of the experimental thermogram allows for the determination of all the parameters for the multiparametric analysis: A_i , $T_{c,i}$, w_i , AUC_n , AP_n , and T_{ave} .

3.2. Characteristics of control samples

We analyzed plasma from 36 healthy subjects (controls) and from 8 patients who had successfully completed surgical management for their melanoma and were shown by continued clinical assessment to maintain no evidence of disease (NED) status (melanoma controls, MC) (see Figure S2). No statistically significant differences ($p < 0.05$) were observed between the groups based on AUC_n , AP_n , and T_{ave} values (Figure S2). This shows that there are no discernable differences between DSC thermograms from healthy controls and from melanoma patients who have no evidence of disease. This is a key observation to validate DSC as a useful approach for melanoma patient monitoring.

3.3. Three-dimensional graphs: control ellipsoid and distance distribution of melanoma patients

A well-defined region corresponding to the cluster of healthy and melanoma controls can be observed after representing AUC_n , T_{ave} , and AP_n values as Cartesian coordinates in a three-dimensional graph (Figure 1A).

The close match between healthy controls and melanoma controls defines a region in the three-dimensional plot that we term “no evidence of disease” (NED), which is the reference

region when comparing the values obtained for melanoma patients in surveillance for disease recurrence. This region can be represented by an ellipsoid centered at $(\overline{AUC_n}, \overline{AP_n}, \overline{T_{ave}})$, with boundaries defined by σ_{AUC_n} , σ_{AP_n} , and $\sigma_{T_{ave}}$.

Plasma samples from 10 melanoma patients were collected at their scheduled oncology surveillance consultations. Between four and eight samples per patient were analyzed. Figure 1B shows all the data from those measurements (63 in total).

Compared to Figure 1A, it can be observed in Figure 1B that some melanoma patient thermograms are located within the control ellipsoid (that is, in the NED region), but some thermograms fall outside this ellipsoid. The goal of this work is to develop a simple and useful approach for monitoring patients and tracking their disease status. A single metric would be convenient for assessing disease status. We calculated the distance of a given thermogram to the NED region and tested the utility of this metric for assessing the clinical status of melanoma patients in this study.

3.4. The d-value: the distance of a thermogram from the NED region

We defined the d-value as the Euclidean distance between the location of a patient thermogram (using AUC_n , AP_n , and T_{ave} as Cartesian coordinates) to the centroid of the control ellipsoid or NED region (see Material and Methods section). Because the values of the three parameters are of different magnitude, the d-value was calculated by normalizing the values of each parameter.

Figure 2 shows a comparison between the d-values of different subject groups. The healthy control group (controls) and the melanoma control group (MC) have been pooled into the “no evidence of disease” controls (NED controls), which is a very homogeneous group.

There is no statistically significant difference between healthy subjects (controls) and melanoma patients ($p > 0.05$) who were clinically determined to have no evidence of disease (melanoma controls, MC). The healthy subjects and melanoma controls were grouped together into the no evidence of disease group (NED control) (Figure 2, left panel).

The d-values for the thermograms from melanoma patients in surveillance were compared to the NED control group. Thermograms from patients with active disease at the time of plasma sample collection are characterized by d-values that are higher than those of the NED control group. A NED region or d-value threshold can be defined from the mean d-value for the NED control group and its standard deviation (Figures 3–5). The d-values for the thermograms from patients with NED status designations are close to those of the NED control group. According to Figures 3–5, d-values do not exceed 5; there are 11 samples with d-values significantly above the control group region (defined as the average control value plus twice its standard deviation); 12 samples with d-values around the control group threshold; and the rest (40 samples) have d-values within the control group region, and typically around the threshold.

Examining the clinical data for the 11 thermograms in the MUSP group with a high d-value, 4 of these 11 thermograms corresponded with plasma samples collected at the beginning of

patient surveillance where patients had recently undergone surgery or just started adjuvant treatment. This could explain why these samples still exhibited d-values in disagreement with the clinical NED status: the metabolic state of blood plasma had not yet been restored from the cancer-associated state. Four other samples of the 11 thermograms with high d-values corresponded to disease recurrence—some of these samples showing early indication of disease recurrence (Patient 7, samples 4–5, and Patient 8, samples 3–4). Only for one patient sample, Patient 9 sample 4, the d-value is above 1 and does not agree with clinical evaluation, but in later visits the d-value reenters the NED region. Overall, this potentially suggests that the DSC thermogram was able to detect metabolic alterations in blood plasma associated with recurrence before the tumor acquired a detectable size for clinical determination.

3.5. Use of DSC thermograms for the monitoring of melanoma patients (10 patients)

The utility of DSC for patient monitoring would lie in the ability of DSC to track the clinical status of a patient in a personalized way. The analysis of the thermogram and clinical results obtained for each case provides relevant information regarding the ability of DSC to detect disease, the comparison between the threshold for detecting disease using diagnostic imaging scans and DSC thermograms, and the agreement between both monitoring tests.

The ten MUSP patients were split into three different categories based on disease status, treatment and corresponding d-value. The five patients within Case A had surgical resection of their melanomas, received adjuvant therapy and had thermogram d-values that corresponded to the clinical determination of disease status. Plasma samples were collected from patients at clinic visits over a period of one to two years and analyzed for this study. Case B patients were placed within the active disease group. Patient 6 had active disease from their initial diagnosis and during treatment. Patients 7 and 8 were initially placed on adjuvant therapy after surgical resection of their melanomas but had disease recurrence. Patient 8 was switched to an alternative treatment regimen. The last group of patients, Case C, had results that were discordant with their clinical status: clinically the patients are like Case A, but the thermogram results were discordant with clinical assessment.

3.5.1. Case A: no evidence of disease (NED) according to both clinical assessment and DSC thermograms

This group comprised stage 2 or 3 melanoma patients considered clinically to have no evidence of disease after surgical resection of their melanomas followed by adjuvant chemotherapy. For each patient, between 6 and 8 plasma samples were analyzed by DSC at different time points spanning before, during and after chemotherapy over a period of one to two years (Figure 3). Comparing thermograms during and after chemotherapy, it is observed that the thermograms were unaffected by the therapy. It should be noted that an initial NED status was determined after clinical resection of the melanomas and was maintained as the clinical status during initial rounds of chemotherapy. This was not confirmed by imaging assessment until the second or third round of chemotherapy or at post-treatment (as indicated in Table S1). By the time of the last sample, none of the four patients exhibited any melanoma recurrence.

Not all DSC thermogram samples have been paralleled with clinical assessment. Clinical assessments and thermograms provided identical results in all samples, except just a minor discrepancy in Patient 5 (sample 6, but d-value is below 1) (Figure 3). Contingency tables for clinical assessment and DSC results are shown in Figure S4. In addition, the few d-values found outside the NED control region mainly correspond to the first initial thermogram in a patient, i.e., initial sample after surgical resection. The interpretation of these results is that there might be a residual altered state as a result of the disease after surgical resection that might not be clinically detectable, but could still be reflected in an altered metabolic state of the blood plasma. This could be a consequence of the high sensitivity of the DSC thermogram for detecting alterations in the composition and interactions in blood plasma. Because it is a minimally-invasive test, the DSC thermogram can be prescribed as many times as required by the physician during patient surveillance to assess when the metabolic state of the blood plasma returns to a normal/healthy profile after clinical treatment. Thus, whenever a DSC thermogram result provides a positive result for melanoma recurrence, subsequent thermograms performed close in time to this sample can be used to confirm a recurrence.

3.5.2. Case B: melanoma disease according to both clinical assessment and DSC thermograms

These patients had active disease during the monitoring period (Figure 4). It is important to note that Patient 6 had an active disease status at the beginning of the treatment, while Patients 7 and 8 had initial NED disease status. Also, Patient 8 was placed initially on adjuvant chemotherapy, but it was switched to alternative therapies due to metastasis later in the treatment.

The results show that DSC analysis detects melanoma disease with high sensitivity (Figure 4). The only sample where clinical assessment and DSC thermogram results do not coincide corresponds to non-radiological clinical assessment. Interestingly, plasma thermograms appear to detect melanoma recurrence before clinical assessment (see Figure S4). For Patients 6 and 7 there is little clinical evidence throughout their treatment history. Thermograms for Patients 6 and 7 were able to detect a change in disease status ahead of clinical determination. This would suggest that DSC can be used to fill the gaps for infrequent radiological assessments and/or unreliable clinical exams. Interestingly, for Patient 8 the first clinical assessment (Sample 3) does not indicate illness, while the DSC thermogram provides an earlier indicator of recurrence, confirmed by a later radiological assessment.

3.5.3. Case C: discordant results between clinical assessment and DSC thermograms

These patients were determined to be NED throughout their clinical surveillance and were placed on adjuvant chemotherapy treatment. Surprisingly, the thermogram results overestimated recurrence for these patients (Figure 5). For Patient 10 the d-values are close to the NED threshold. We must take into consideration that if this method is used for patient monitoring, the threshold could be set individually for each patient. For Patient 10, if the NED threshold was set slightly higher, the thermogram results would concur with clinical assessment. For Patient 9 a similar scenario is found, although there is a sample with larger

discrepancy (sample 4). At the end of the surveillance period no melanoma recurrence was observed for both patients. These two patients (9 and 10) provide the more discrepant results between clinical assessment and DSC thermogram. There might be an overestimation of tumor recurrence in DSC (sometime due to the sensitivity of the technique, or due to incomplete remission of the altered metabolic state in plasma), but the DSC test can be repeated with no risk and at low cost. It might be better to have false positives than false negatives, and according to the contingency tables (Figure S4), there are no false negatives provided by the DSC tests.

4. Discussion

Due to rising health care costs and limited effectiveness for low stage patients, there is a need for new surveillance methods for melanoma patients that are cheaper, faster and low-risk to fill the gap that diagnostic imaging and clinical assessments cannot achieve. Differential scanning calorimetry (DSC) has been shown to detect differences in blood serum proteome/interactome in a variety of diseases [17–23]. Serum thermograms provide information about the metabolic state (protein composition, metabolites, altered biomolecular interactions) and can be used as a reasonably quick, minimally-invasive, low-cost surveillance method as a complement to current diagnostic approaches. From our previous work, we described the value of combining multiparametric analysis with DSC providing a multitude of graphical and numerical tools that can be used to quantify differences in thermogram data. Therefore, the purpose of this study was twofold: to apply our robust multiparametric analysis to a subset of melanoma patients and to compare subsequent thermogram values with time series clinical data in order to assess the utility of the methodology for patient monitoring.

To test the correlation of DSC analysis with clinical reference data, time series clinical data were compared to the d-values for each MUSP patient (Figures 3–5). Each sample had an accompanying clinical designation of NED/Active/Recurrence with some samples having accompanying status determinations (e.g. radiology) that were used as validation for the disease status designations. Comparing these status determinations with the d-values, we found that our thermogram results corresponded well with the clinical assessment of the disease status state, and even for some patients recurrence was detected early (Patients 6 and 7). For both patients only clinical assessments (i.e., no radiology or biopsy assessments) or no assessments were done prior to finding a recurrence through thermograms. This would suggest that DSC has high utility for early detection of recurrence and might be more reliable than clinical assessment as a diagnostic test. While some false positives were found these were mostly associated with initial samples after surgical resection, where the metabolic state of the plasma may still reflect the presence of residual disease. It is important to note that in all the cases studied in this work, no false negatives were obtained by DSC thermograms, taking as a reference the clinical (radiological or non-radiological) assessment. Thus, higher sensitivity and lower threshold in DSC might allow detecting metastasis and recurrence in patients earlier. For the majority of cases, there is a correlation between the clinical assessment and the multiparametric DSC analysis.

Although this is a small, limited study ($n = 10$, with 63 total samples analyzed), the results are promising and support future work involving a larger sample set to determine the sensitivity and specificity of DSC thermograms for detection of recurrences in the melanoma setting. On the other hand, there are some patients with high d-values for early MUSP visits. In some cases the high d-value may be due to an early DSC determination after surgery where the altered plasma metabolic state has not returned to a healthy state. But, still this could be an indication of the initial state of the patient after surgery, and this may be taken as a personalized reference for the subsequent surveillance. Therefore, future work remains to apply our procedure to a larger set of melanoma patients and to investigate the plasma state at early visits after resection for melanoma patients.

DSC exhibits a great advantage as a monitoring approach. The thermogram from a patient at a certain time can be compared with some indexes or parameters from a control group, in order to detect significant differences. In addition, by performing thermograms close in time it is possible to elaborate a personalized monitoring procedure based on the patient's history, taking her/his own reference control values, without the need to include external controls, in order to detect alterations in the patient's serum.

From the contingency tables (Figure S4), it can be observed there are no cases in which DSC thermograms provide a false negative, i.e., a negative result when the clinical radiological assessment indicates tumor existence. Therefore, DSC thermograms detect all the tumors that have been detected by imaging scans. In addition, there is ~80% agreement between both type of tests, clinical assessments and DSC thermograms. The only discrepancies correspond to melanoma recurrences observed by DSC thermograms, which can be reaffirmed or rejected in subsequent analyses.

There is a need for new surveillance tools (e.g. cheaper, faster, lower risk) for melanoma patients that would allow patient monitoring with lower potential side effects and increased patient adherence. This work represents a proof of principle for implementing Thermal Liquid Biopsy (DSC thermogram analysis of serum samples) as a valuable approach for a personalized diagnostic assessment of melanoma patients. This methodology would allow physicians a closer and personalized monitoring protocol to help in assessing patient health status and to facilitate the decision-making process. Combining thermograms with the multiparametric analysis allows for definitive single diagnostic values to be obtained for comparing with the control reference values. This combined technique can be run congruently with current imaging modalities by performing routine imaging as currently prescribed (e.g. 2–4 times a year) while having more frequent thermogram DSC tests done (e.g. monthly or bimonthly). The advantages of Thermal Liquid Biopsy are paramount: reducing the monitoring time interval without a significant increase in economic cost or burden/risk for the patient, while providing diagnostic value for a patient's current disease state.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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LIST OF ABBREVIATIONS

DSC	Differential Scanning Calorimetry
CT	Computed Tomography
GAC	Gastric Adenocarcinoma
IRB	Institutional Review Board
MUSP	Melanoma Under Surveillance Patients
MC	Melanoma Controls
NED	No Evidence of Disease
C_p	Heat Capacity
T	Temperature
A	Height of the peak
T_c	Center of the peak
w	Width of the peak
AUC	Area Under the Curve
AP_n	Area of the normalized heights polygon

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HIGHLIGHTS

- Thermal Liquid Biopsy is a blood test that can be easily and routinely performed
- This test can be applied routinely during the surveillance of melanoma patients
- Thermal Liquid Biopsy can be used to complement standard clinical assessment
- This methodology can provide a personalized diagnostic assessment of patient health
- Thermal Liquid Biopsy can aid physicians in the clinical decision-making process

Figure 1.

(A) AUC_n , T_{ave} , and AP_n values from plasma thermograms of healthy controls (black squares) and melanoma controls (red squares) in a Cartesian three-dimensional plot (XYZ representation, left, and XYZ projections, right). (B) AUC_n , T_{ave} , and AP_n parameters from plasma thermograms of NED controls (green squares), and melanoma patients under surveillance (orange squares) in a Cartesian three-dimensional plot (XYZ representation, left, and XYZ projections, right). Data from the thermograms of 10 melanoma patients at different time points (see Table 1 and Table S1) were obtained, with a total of 63 thermograms. Each orange square corresponds to the thermogram of a given patient at a certain time point.

Figure 2. d-values from all plasma thermograms in this study. **(Left panel)** Expanded view for the d-values for healthy controls (black squares), melanoma controls (MC; red squares), and both healthy and melanoma controls grouped together as NED controls (green squares). There is no statistically significant difference ($p > 0.05$) between both groups (healthy controls and MC). **(Right panel)** d-values for NED controls (green squares), melanoma under surveillance patients (MUSP) (orange squares) and the MUSP subgroups, active disease patients confirmed by clinical or radiological diagnosis (filled orange circles) and patients determined clinically or radiologically to have no evidence of disease (open orange circles). In the left panel, the d-values have been calculated considering the average value of the parameters within the corresponding group. In the right panel, the d-values for the MUSP group (orange squares) have been calculated considering the average value for the parameters for the NED control group.

Figure 3:

Longitudinal comparison of thermogram results and clinical assessments of MUSP plasma samples. Squares correspond to d-values of each DSC thermogram from the sample collected at a certain time after diagnosis. Green squares indicate negative Liquid Thermal Biopsy (LTB) result, i.e., within the NED d-value region (mean value of NED control group plus twice the standard deviation, yellow shading) and red squares indicate positive LTB result, i.e., outside this area. Arrows correspond to clinical assessment performed at a certain time after diagnosis. Solid arrows represent a radiological assessment and dashed arrows represent a clinical assessment. Green or red arrows represent NED or active disease, respectively.

Figure 4:

Longitudinal comparison of thermogram results and clinical assessments of MUSP plasma samples. Squares correspond to d-values of each DSC thermogram from the sample collected at a certain time after diagnosis. Green squares indicate negative LTB result, i.e., within the NED d-value region (mean value of NED control group plus twice the standard deviation, yellow shading) and red squares indicate positive LTB result, i.e., outside this area. Arrows correspond to clinical assessment performed at a certain time after diagnosis. Solid arrows represent a radiological assessment and dashed arrows represent a clinical assessment. Green or red arrows represent NED or active disease, respectively.

Figure 5:

Longitudinal comparison of thermogram results and clinical assessments of MUSP plasma samples. Squares correspond to d-values of each DSC thermogram from the sample collected at a certain time after diagnosis. Green squares indicate negative Liquid Thermal Biopsy (LTB) result, i.e., within the NED d-value region (mean value of NED control group plus twice the standard deviation, yellow shading) and red squares indicate positive LTB result, i.e., outside this area. Arrows correspond to clinical assessment performed at a certain time after diagnosis. Solid arrows represent a radiological assessment and dashed arrows represent a clinical assessment. Green or red arrows represent NED or active disease, respectively.

Table 1.

Demographic characteristics and clinical status of the participants

	Control Subjects (n=36)	Melanoma Controls (n=8)	Melanoma Patients in study (n=10)
Age (years) Mean (sd)	34 ± 11	51 ± 10	51 ± 15
Sex			
<i>Male</i>	19 (53%)	4 (54%)	4 (40%)
<i>Female</i>	17 (47%)	4 (46%)	6 (60%)
Ethnicity			
<i>Caucasian</i>	22 (61%)	7 (87%)	10 (100%)
<i>Black</i>	13 (36%)	1 (13%)	
<i>Hispanic</i>	1 (3%)		
Total of samples studied per group	36	13	62
Melanoma Disease			
<i>NED*</i>	--	8 (100%)	7 (70%)
<i>Active Disease</i>		--	3 (30%)
Melanoma Treatment			
<i>During study</i>		--	10 (100%)

* = No Evidence of Disease